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Endocrine disruption and detoxification processes provoked by low levels of pollutants in cultured fish

TESIS DOCTORAL

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Endocrine disruption and detoxification processes provoked by low levels of pollutants in cultured fish

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Es curioso cómo la parte que debería ser más fácil (y la que más ganas tenía de escribir), es la que más me está costando y para la que no encuentro las palabras…

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List of abreviations

11-KT	11-ketostestosterone
ABC	ATP-binding cassette
ABM	Active biomonitoring
AhR	Aryl hydrocarbon receptor
ANOVA	Analysis of Variance
ANOVA	Analysis of Variance
APC	Antigen presenting cell
AR	Androgen receptor
ARE	Androgen response element
ARNT	Aryl hydrocarbon receptor nuclear translocator
BaP	benzo(*a*) pyrene
BEQs	Bio-equivalents
BFCOD	Benzyloxy-4-[trifluoromethyl]-coumarin-*O*-debenzyloxylase
BPA	Bisphenol A
CRH	Corticotrophin-releasing hormone
CYP	Cytochrome p450
D1	Deiodinase type I
D2	Deiodinase type II
D3	Deiodinase type III
DDT	Dichlorodiphenyltrichloroethane,
DHT	Dihydrotestosterone
DMEs	Drug metabolizing enzymes
DNA	Deoxyribonucleic Acid
DRE	Dioxin response elements
E1	Estrone
E2	17 β-estradiol
E3	Estriol
EC50	Half effect concentration
EDCs	Endocrine disrupting compounds
EE2	17α-ethinyl oestradiol
ER	Estrogen receptor
ERE	Estrogen response elements
EROD	Ethoxyresorufin-*O*-deethylase
FSH	Follicle Stimulating Hormone
FSHR	Follicle Stimulating Hormone Receptor

GCxGC-TOF-MS	Two-dimensional gas chromatography–time-of-flight mass spectrometry
GnRH	Gonadotropin Releasing hormone
GnRHR	Gonadotropin Releasing hormone receptor
GSTs	Glutathione-S-transferases
GTH	Gonadotropin
HEK-293	Human embrionic kidney cell line
HER-LUC	HEK-293 cell line stably transfected with sea bass ERα (sbERα) and luciferase.
HPG	Hypothalamus-pituitary-gonadal axis
HPT	Hypothalamus-pituitary-thyroid axis
HRE	Hormone response elements
HRGC/HRMS	Gas chromatography/high-resolution mass spectrometry
HSD	Hydroxysteroid dehydrogenases
IEH	Institute for Environment and Health
IFN	Type I interferon
Ig	Immunoglobulin
IL	Interleukin
IRD	Inner ring deiodination
LBDs	Ligand binding domains
LH	Luteining hormone
LHR	Luteining hormone Receptor
LPS	Lipopolysaccharide
MAPK	Mitogen –activated protein kinase
MMI	Methimazole
mRNA	Messenger RNA
MRP	Multidrug resistance-associated protein
NcoR	Nuclear receptor co-repressor
NIS	Sodium/iodide symporter
NR	Nuclear receptor
OC	Organochlorine
OECD	Organization for Economic Cooperation and Development
OHA	11B-hydroxyandrostenedione
OHT	11B-hydroxytestosterone
ORD	Outer ring deiodination
PAHs	Polycyclic aromatic hydrocarbons
PBDEs	Poly brominated diphenyl ethers

PCBs	Polychlorinated biphenyls
PCDD/Fs	Dibenzo-p-dioxins/polychlorinated dibenzofurans
PC-DR-LUC	P12 cell line stably transfected with the avian THRα (avTHRa) and luciferase.
PCPs	Personal Care Products
PTU	6-propyl-2-thiouracil.
PXR	Pregnane-X-receptor
RAA	Relative agonistic activity
RAR	Retinoic acid receptors
REACH	European Union Regulation on the Registration, Evaluation, Authorisation and Restriction of Chemicals
RNA	Ribonucleic acid
RTA	Relative transactivation activity
RTG-2	Fibroblast-like cell line from rainbow trout gonad.
RT-qPCR	Reverse transcriptase-quantitative polymerase chain reaction
RXR	Retinoid X receptor
SEM	Standard error of the mean
SHBG	sex hormone binding globulin
SMRT	Silencing mediator for RAR and THR
ßNF	ß -naphthoflavone
SULTs	Sulfo-transferases
T	Testosterone
T3	3,3',5-triiodo-L-thyronine
T4	L-thyroxine
TBBPA	Tetrabromobisphenol A
Tc	Cytotoxic T cell
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TCR	T Cell Receptor
TEF	Toxic Equivalence Factor
Th	Helper T cell
THR	Thyroid Hormone Receptor
THs	Thyroid Hormones
TNF-α	Tumor Necrosis Factor α
TPO	Thyroid peroxidase
TRE	Thyroid response elements
TRH	Thyrotropin releasing hormone
TSH	Thyroid stimulating hormone

TTR	Transthyretin
UGTs	Glucuronyl transferases
VTG	Vitellogenin
WFD	Water Framework Directive
WHO	World Health Organization
WWTP	Wastewater Treatment plant
XRE	Xenobiotic response elements
YES	Yeast screen assay

Summary

Water is undoubtedly the most important resource on Earth, since it is an essential element for the development of life. However, human being has exerted a negative impact onto its quality and currently almost no waterbody can be considered absolutely pristine. With the exceptions of those cases of severe contamination, organisms in nature are not exposed to high concentrations of a single pollutant, but on the contrary to mixtures of hundreds or thousands of compounds present at low concentrations (ng/L). These are the so-called micropollutants and currently the assessment of their occurrence and effects represents a major challenge not only to scientists but also to policymakers. Among them, the "endocrine disrupting compounds" (EDCs), this is, those substances able to interfere with the endocrine system of an organism or its progeny, are of special concern as they can provoke deleterious effects at very low concentrations. Provided the fine-tuning of the hormonal systems, which control key processes such as development and reproduction, any disturbance may lead to dramatic consequences including the collapse of populations. Although any organism is susceptible of being affected by such compounds, fish are especially sensitive as they are immersed throughout their whole lifespan in the aquatic medium, which is the fate of any kind of pollutant (due to direct dumping or spillage, to deposition from the air, or to leakage or runoff from soils). into account that fish are aquatic organisms with long lifecycles, on top of the food chain and with a high economic value, the assessment of the effect of micropollutants and EDCs on their health is of great importance. However, although feral fish offer a great number of advantages in monitoring environmental pollution, they also present a series of disadvantages, including the ability to migrate all along a particular water body or the generation of resistance mechanisms. As an alternative, the use of farmed fish as sentinels of pollution has arisen as a possibility. There, fish are maintained in controlled conditions and continuously exposed to the micropollutants which may be present in the waters. It is necessary to note that in this type of facilities, feeds may represent another potential source of pollutants which needs to be evaluated and characterized.

Therefore, taking into account the above mentioned, the objective of this thesis was to evaluate from an integrative perspective the presence of micropollutants, including EDCs, on water bodies through their effects onto farmed fish. In order to do so, we identified the two main potential sources of exposure (water and feed) and organized the work around these sources. In this way, Chapter I (Research articles I and II) deals with the detection and evaluation of micropollutants and EDCs in waters. To this aim, we performed two sampling campaigns in two rainbow trout (*Oncorhynchus mykiss*) farms and applied a methodology based on the combination of biological and chemical tools to evaluate the presence and effects of

micropollutants in fish. In each sampling, biomarkers of environmental pollution (Cytochromes p450 1A and 3A, CYP1A and CYP3A, respectively) were measured in the liver of sampled fish both at the transcriptional and enzymatic levels. CYP1A, which is induced after ligand-activation of the aryl hydrocarbon receptor (AhR), was measured as ethoxyresorufin-*O*-deethylase (EROD) activity while CYP3A was assessed by means of benzyloxy-4-[trifluoromethyl]-coumarin-*O*-debenzyloxylase (BFCOD) activity. The presence of AhR agonists in sediments was assessed *in vitro* by measuring EROD activity in a fibroblast-like cell line originated from rainbow trout gonadal tissue(RTG-2). In addition, sediment and water samples were collected and analyzed by means of two-dimensional gas chromatography–time-of-flight mass spectrometry (GCxGC-TOF-MS), which evidenced the ubiquitous presence of low levels (ng/L) of polycyclic aromatic hydrocarbons (PAHs) and personal care products (PCPs). A strong and time limited EROD induction in one of the fish farms motivated the use of another approach: Active Biomonitoring (ABM), which consists on the transference of some animals to clean waters monitoring the behavior of the measured biomarkers. After only seven days in a farm with controlled conditions, we observed a significant reduction in the induced enzymatic activities. Collectively these results suggest the presence of low levels of contaminants that can be detected only through the appropriate combination of biological and chemical techniques..

In Chapter II (Research Article III), we aimed to assess the presence of EDCs in commercial fish feeds by means of three *in vitro* assays. More specifically we first tested the potential AhR agonistic activity by measuring EROD activity in the RTG-2 cell line. Secondly, in order to assess the potential estrogenic activity present in the feed pellets we developed and validated a new reporter assay, named HER-LUC. This assay is based on the use of a cell line (HEK-293) stably transfected with the sea bass estrogen receptor α (sbERα) and the luciferase gene under the control of estrogen responsive elements (ERE). Thirdly, we evaluated the potential thyromimetic activity of the fish feeds with a cell line, PC-DR-LUC, stably transfected with an avian thyroid receptor (THR) and the luciferase gene as reporter gene under the control of the mentioned receptor (Jugan et al., 2007). Our results showed a relatively low estrogenicity but surprisingly, and for the first time, a high thyromimetic activity which motivated the third part of this work. Considering this high thyromimetic activity in fish feeds, together with the increasing evidence linking thyroid and immune systems in mammals, we wanted to explore if such relationship also applies to piscine organisms. In this study, presented in Chapter III (Research Article IV) we first characterized the presence of THRs in immune organs and cells of juvenile rainbow trout. We demonstrated that immune organs (head kidney and spleen) and isolated immune cells (from head kidney and peripheral blood) of rainbow trout express both thyroid receptor α (THRA) and

β (THRB), indicating that thyroid signaling is possible in the immune system of this species. The higher expression of THRA in immune-related tissues in comparison to the reference tissue (liver) suggested that this subtype would be the main mediator of thyroid hormones (THs) on trout immune cell function. Thereafter, we performed an in vivo experiment in which juvenile trout were exposed to the active thyroid hormone triiodothryronine (T3) or to the anti-thyroid drug propylthiouracil (PTU) for 15 days. Both treatments altered THR expression in immune organs and cells although in a tissue-dependent manner. Lastly, to explore whether changes in thyroid status affect leukocyte population composition, several marker genes of macrophages, T lymphocytes and B lymphocytes, were measured. Our results showed that the effects of T3 and PTU treatments on leukocyte marker genes were also tissue- and time-dependent.

Resumen

El agua es, sin lugar a dudas, el recurso más importante de la Tierra puesto que es un elemento indispensable para el desarrollo de la vida. Sin embargo, la acción del hombre ha ejercido un impacto negativo sobre su calidad lo cual ha provocado que, actualmente, prácticamente ningún sistema acuático puede ser considerado como absolutamente prístino. Con la excepción de aquellos casos de contaminación severa, normalmente los organismos en la naturaleza no se encuentran expuestos a grandes concentraciones de un solo contaminante, sino que, al contrario, están expuestos a mezclas de cientos o miles de compuestos en bajas concentraciones (ng/L). Son los llamados "microcontaminantes", cuya presencia y efectos suponen un gran desafío no solo para los científicos sino también para los reguladores. Dentro de éstos, los denominados "disruptores endocrinos" (DEs, del inglés "endocrine disruptor", aunque en castellano se podría hablar de "alteradores" o "perturbadores endocrinos")son causa de especial preocupación, ya que son capaces de interferir con el sistema endocrino de los organismos o de su progenie causando efectos muy sutiles a concentraciones extremadamente reducidas.Puesto que el sistema endocrino de los organismos regula procesos clave tales como el desarrollo o la reproducción está sometido a un estricto control y regulación fisiológica. El resultado es que cualquier pequeña alteración de esa regulación puede llevar a un malfuncionamiento con consecuencias dramáticas para el organismo e, incluso, para las poblaciones. A pesar de que cualquier organismo es susceptible de verse afectado por dichas sustancias, los peces son especialmente sensibles ya que se encuentran en un medio, el acuático, que constituye el destino de cualquier contaminante (por vertido directo, por deposición desde el aire, o por escorrentía o lixiviación desde los suelos). Teniendo en cuenta que son organismos con largos ciclos de vida, en la cúspide de la cadena alimenticia y con un gran valor económico, la evaluación de los efectos de microcontaminantes y DEs sobre su salud es de especial relevancia. Sin embargo, aunque los peces salvajes ofrecen una serie de ventajas con vistas a la monitorización de contaminantes en el medio ambiente, también presentan una serie de desventajas, incluyendo la habilidad de migrar dentro de un determinado cuerpo de agua o la generación de mecanismos de resistencia. Una alternativa factible y muy adecuada puede ser el uso de peces cultivados en piscifactorías. En ellas, los peces se encuentran en condiciones controladas y continuamente expuestos a los microcontaminantes que puedan estar presentes en las aguas. Es necesario mencionar que en este tipo de instalaciones los piensos con los que se alimenta a los animales pueden representar otra fuente de contaminación, la cual debe ser evaluada y caracterizada.

Teniendo en cuenta lo mencionado previamente, el objetivo de esta tesis fue evaluar, desde una perspectiva integradora, la presencia de microcontaminantes, incluyendo Des, en cuerpos de agua a través de sus efectos sobre los peces cultivados. Para ello, se identificaron las dos fuentes principales de exposición (agua y alimento) y se organizó el trabajo alrededor de esas dos fuentes. El Capítulo I (Artículos I y II), trata sobre la detección y evaluación de los microcontaminantes y DEs en las aguas. Con este fin, se realizaron dos campañas de muestreo en dos piscifactorías de trucha arcoíris (*Oncorhynchus mykiss*) y se aplicó una metodología basada en la combinación de herramientas biológicas y químicas para evaluar la presencia y efectos de dichos microcontaminantes sobre los peces. En cada muestreo, se midieron biomarcadores de contaminación ambiental (citocromos p450 1A y 3A, CYP1A y CYP3A, respectivamente) tanto a nivel transcripcional como a nivel enzimático. El CYP1A, que se induce tras la unión de un ligando al receptor de hidrocarburos aromáticos (AhR), se midió como actividad etoxirresorrufina-*O*-deetilasa (EROD) mientras que el CYP3A se evaluó por medio de la actividad benziloxi-4-[trifluorometil]-cumarin-*O*-debenciloxilasa (BFCOD). La presencia de agonistas del AhR en sedimentos se evaluó *in vitro*, midiendo la actividad EROD en la línea celular RTG-2, una línea tipo fibroblasto procedente de tejido gonadal de trucha arcoíris. Además, se tomaron muestras de sedimento y de agua y se analizaron por medio de cromatografía de gases bidimensional acoplada a tiempo de vuelo y espectometría de masas (GCxGC-TOF-MS), evidenciándose la presencia ubicua de niveles bajos (ng/L) de hidrocarburos poliaromáticos (PAHs) y productos de cuidado personal (PCPs). Una inducción fuerte y puntual de actividad EROD en una de las piscifactorías, motivó el uso de otra aproximación: la biomonitorización activa (ABM), la cual consiste en la transferencia de algunos animales a aguas limpias con el objetivo de monitorizar el comportamiento de los distintos biomarcadores. Tras siete días en una piscifactoría con condiciones controladas, observamos una reducción significativa en las actividades enzimáticas. Colectivamente, esos resultados sugieren la presencia de niveles bajos de contaminantes que causan efectos muy sutiles y que solamente se pueden detectar mediante la apropiada combinación de técnicas biológicas y químicas .

El capítulo II (Artículo III), tiene como objetivo evaluar la presencia de DEs en piensos comerciales por medio de tres ensayos *in vitro*. Primeramente se evaluó la presencia potencial de agonistas del AhR, para lo cual se midió la actividad EROD en la línea celular RTG-2. En segundo lugar, con el objetivo de evaluar la potencial carga estrogénica presente en los piensos, desarrollamos y validamos un nuevo ensayo, llamado HER-LUC. Este ensayo está basado en el uso de una línea celular (HEK-293) transfectada de manera estable con el receptor de estrógenos α de lubina (sbERα) y el gen de la luciferasa bajo el control de elementos de respuesta a

estrógenos (ERE). En tercer lugar, evaluamos la potencial actividad tiromimética de los piensos mediante una línea celular, PC-DR-LUC, transfectada de manera estable con un receptor de tiroideas (THR) aviar y con luciferasa como gen informante bajo el control del THR (Jugan et al., 2007). Nuestros resultados mostraron una relativa baja estrogenicidad pero sorprendentemente, y por primera vez, una alta actividad tiromimética, lo cual motivó la tercera parte de este trabajo. Teniendo en cuenta la alta actividad tiromimética observada en peces y las observaciones realizadas en mamíferos que demuestran una influencia del sistema tiroideo sobre la función inmune, quisimos observar si tal influencia se da también en peces. Para ello realizamos un cuarto estudio presentado en el capítulo III (Artículo IV). Primeramente caracterizamos la presencia de THRs en órganos y células inmunes de juveniles de trucha arcoíris. Demostramos que tanto los órganos inmunes (riñón anterior y bazo) como las células inmunes aisladas (del riñón anterior y de la sangre) de la trucha arcoíris expresan los receptores de hormonas tiroideas alfa y beta (THRA y THRB, respectivamente), indicando que una señalización del sistema tiroideo es posible en el sistema inmune de esta especie. La mayor expresión del THRA en los órganos y células inmunes en comparación con el órgano usado como referencia (hígado), sugiere que este subtipo sería el principal mediador de la acción de las hormonas tiroideas (THs) sobre la función inmune de la trucha. Posteriormente, realizamos un experimento in vivo en el cual juveniles de trucha fueron expuestos a la hormona tiroidea activa triiodotironina (T3) o a la droga anti-tiroidea propylthiouracil (PTU) durante 15 días. Ambos tratamientos alteraron la expresión de los THRs en los órganos y células inmunes, aunque las modificaciones fueron más o menos acusadas dependiendo del tejido estudiado. Por último, con el objetivo de explorar si los cambios en el estatus tiroideo afectan a la composición de las poblaciones de leucocitos, se midieron varios genes marcadores de macrófagos, linfocitos T y linfocitos B. Nuestros resultados mostraron que los efectos de la T3 y del PTU sobre los genes marcadores dependen tanto del tejido estudiado como como del tiempo.

The Origin of the Ecotoxicology

Pollution is an environmental problem inherent to human development, and has been present along the history in a variety of forms. Indeed, although it may seem a modern issue, human being has been affecting the quality of the surrounding environment since the beginning of time. The presence of soot in prehistorical caves can be regarded as the first evidence of environmental pollution (Makra and Brimblecombe 2004). Other examples of historical contamination include, for instance, the recent finding of lead (Pb) pollution in Keweenaw Peninsula lake sediments dating back at least 8,000 years, and which have been associated with early copper mining in North America (Pompeani et al. 2013).

However, despite of environmental pollution being coeval to human history, it has been in the last century when the environment's quality has suffered a more abrupt degradation, surely as a consequence of the great increment of human activity in a number of fields including industry, agriculture and medicine. There has been a parallel scientific-technical progress, which has led to an undeniable improvement in the quality of life of millions of people worldwide, but it has also triggered the exponential growth of new synthesized and marketed chemicals that have been released to the environment. The CAS REGISTRY (www.cas.org), the largest database on chemical substance information, currently includes over 91 million of organic and inorganic different substances and it is enlarged at an approximate rate of 15,000 substances/day (February 2015). Although of those only a small number is actually being marketed and introduced in the environment (Binetti et al. 2008), the global chemical production is projected to grow at an enormous rate: 3% annually; soon surpassing the global population growth (Fig. 1) (Wilson and Schwarzman 2009). This will generate great benefits but also will lead to a decrease of environmental quality worldwide(Wilson and Schwarzman 2009).

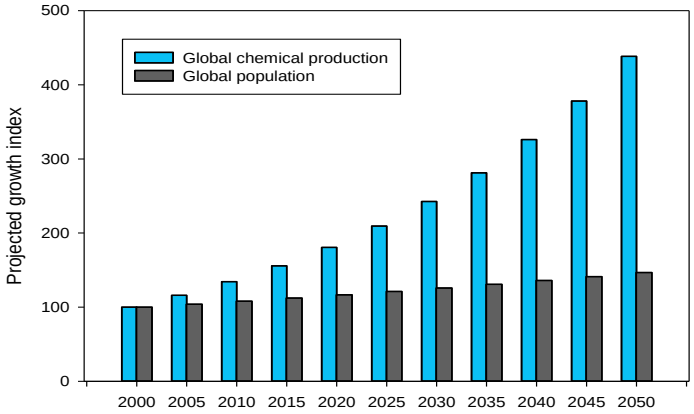


Fig. 1 Comparison of global chemical production (blue), projected to grow at a rate of 3% per year and global population growth (grey), estimated at 0.77% per year. American Chemistry Council 2003; OECD 2001; United Nations 2004. Modified from Wilson and Schwarzman, 2009.

As a consequence, the scientific community first, and the governments and regulatory agencies later, have started to show their concern on the effect of those chemicals over organisms and ecosystems. In this context, the publication in 1962 of “Silent Spring”, by Rachel Carson (Carson, 1962) was a milestone and ever since has been considered as a referent in this field. This increasing interest on the study of the effects of chemicals over organisms (other than humans) led to the introduction of the term “Ecotoxicology” in 1969 by Truhaut. Defined then as “the branch of toxicology concerned with the study of toxic effects, caused by natural or synthetic pollutants, to the constituents of ecosystems, animal (including human), vegetable and microbial” (Truhaut 1977) outlines an integrative approach. In this sense, Ecotoxicology can be considered as a multidisciplinary subject which integrates toxicology and ecology.

Eventough researchers were able to describe toxic effects of chemicals on organisms as early as the mid 40´s (Grindley 1946, Pielou 1946, Ginsburg 1947), it was not until 30 years later that governments started to take action. The adoption in the U.S of the Toxic Substance Control Act (TSCA) in 1976, can be regarded as the first governmental attempt to regulate chemicals. Not including fuels, pesticides products, pharmaceuticals or food products which are regulated by additional laws, TSCA currently lists around 83,000 marketed substances. On the other hand, European Union’s REACH (Regulation of the European Parliament and the Council concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals) (EC, 2006), enforced on June 2007, initially outlined the ambitious expectation of filling the knowledge gaps for a high number of chemicals (about 100,000). This estimation has been surpassed and only in the preregistration phase, now completed, about 145,000 substances have been included. This means that although efforts are being done towards ensuring a higher level of protection of human and environmental health from the chemical-associated risks, still mankind must face great challenges including improving detection and quantification techniques, assessing the combined effects of complex mixtures and developing efficient removal systems.

A.Q-G.

I. INTRODUCTION

1. TYPES OF POLLUTANTS: MACRO vs. MICRO.

According to the Water Framework Directive, 'Pollution' is defined as "*direct or indirect introduction, as a result of human activity, of substances or heat into the air, water or land which may be harmful to human health or the quality of aquatic or terrestrial ecosystems (…)"* (EC, 2000). On the contrary, the term 'Contamination' is commonly defined as "*the presence of elevated concentrations of substances in the environment above the natural background level for the area and for the organism*", this is, it does not imply adverse effects on biota. Therefore, here we will refer only to pollutants and will avoid other terms. In this context, it is necessary to clarify that not all chemicals are pollutants pollutants but on the contrary, a pollutant is only defined as so if it poses a threat to the human or ecosystem´s health. According to the EUROSTAT, more than 50% of the 325 million tonnes of chemicals produced in the EU in 2012 can be defined as toxic (http://epp.eurostat.ec.europa.eu/tgm/table.do?tab=table&plugin=1&language=en&pcode=tsdph320), and thus could be potential pollutants.

Among "Pollutants" it is possible to distinguish macro and micropollutants.

Macropollutants are a small group of naturally occurring compounds present in the environment in an unusual high concentration (from µg/L to mg/L range). These include compounds such as acids, salts, nutrients (phosphorus, P and nitrogen, N) and organic matter and, although they posed an environmental threat in the earlies 70s, their source and behavior are nowadays really well understood and managed (Schwarzenbach et al. 2006).

On the contrary, micropollutants are defined as those substances, both of anthropogenic and natural origin, which are present in the waters (or in the environment) at low or trace concentrations (usually in the ng/L or µg/L range)(Schwarzenbach et al. 2006). Under this term a vast array of substances is encompassed, including pharmaceuticals, personal care products, manufactured nanomaterials, hormones, and pesticides among others. The great diversity of compounds, having different toxicities, chemical structures and properties, together with the fact that they are usually present in complex mixtures at very low concentrations implies a challenge in terms of detection, quantification and toxicity risk assessment. In addition, the lack of proper removal systems in wastewater treatment plants (WWTPs), which are not specifically designed to eliminate such substances, and moreover, the vague regulation concerning water quality standards for most of them, has contributed to the worldwide occurrence of

micropollutants in the aquatic environment (Luo et al. 2014). In this context and given the challenge offered by micropollutants, in this work we will focus on them.

1.1 Types and sources of micropollutants

Although each author describes different categories (Luo et al. 2014, Burkhardt-Holm 2011, Stuart et al. 2012), in general terms, all micropollutants fall within the following five: pharmaceuticals, personal care products (PCPs), hormones, industrial chemicals and pesticides(Burkhardt-Holm 2011). Here, pesticides are considered in a broad sense and include plant protection products and biocides. Another class of micropollutant, only considered very recently as such, are manufactured nanomaterials, which are being increasingly used and introduced in a variety of products. Only in the last years, the release and presence in the environment of some nanomaterials has been reported (Farré et al. 2010, Westerhoff et al. 2011). However, given their particular mechanisms of action not caused by the molecules but by the nanoparticules making them up, they fall out of the scope of this work. Micropollutants may enter the environment in a variety of forms but primarily they are derived from three main sources: domestic wastewater, agricultural and cattle activity and industrial activity.

Industrial and domestic water usage accounts for 10% of the globally accessible runoff and generates a stream of wastewater that ends up in the aquatic environment. Although some control systems such as WWTPS have been established in an attempt to palliate water pollution, the removal efficiency varies considerably not only among pollutants but also among plants (Reviewed in Luo et al. 2014). This meaning that if insufficiently treated, WWTPs effluents can be a major route of surface water contamination with micropollutants (Kasprzyk-Hordern et al. 2009). Agriculture is a diffuse source of pollution with tons of pesticides, veterinary drugs and fertilizers arriving to rivers and lakes by runoff (Schwarzenbach et al. 2006). Other secondary sources of pollution include, for instance, landfill leaching derived from improper disposal or accidental spillages. Table 1 summarizes the main categories and sources of micropollutants, selected relevant examples and concentration range described in the literature.

Pharmaceuticals

Although the presence of pharmaceuticals in water and sediment samples has been only recently reported, they show a widespread distribution in the environment (Behera et al. 2011, Carmona et al. 2014, Moreno-Gonzalez et al. 2014, Wu et al. 2014). Some of the most commonly found compounds are analgesics and anti-inflammatory drugs (i.e.: ibuprofen, diclofenac), anticonvulsants (carbamazepine), β-blockers (i.e.: atenolol, metopropol) and antibiotics (i.e.:

erythromycin sulfamethoxazole) (Table 1), whose environmental concentrations have been found to correlate well with their consumption (Rev. in Luo et al. 2014).

The major routes by which they enter the environment are through excretion, disposal and agricultural usage (Fent et al. 2006), meaning that the main sources are agricultural/cattle activity and domestic wastewater, with hospitals´ effluents having an important impact. Since pharmaceuticals, continuously released in the environment, are designed to elicit specific biological effects in humans at low doses, their occurrence, even at low concentrations, is of high environmental concern (Monteiro and Boxall 2010).

Table 1 Main categories, selected examples and main sources of micropollutants.

Category	Selected examples	Main sources
Pharmaceuticals	*Antiinflamatory* Ibuprofen Diclofenac *Beta –blockers* Atenonol Metoprolol *Antibiotics* Ciprofloxacin Erythromycin	• Domestic wastewater • Cattle and aquaculture activity
Personal care products	*Polycyclic musks* Galaxolide Tonalide *Antimicrobial agents* Triclosan	• Domestic wastewater
Hormones	*Natural hormones* 17ß-estradiol Estrone *Synthetic hormones* 17α-ethynilestradiol Progestin	• Domestic wastewater • Agricultural, cattle and aquaculture activity
Industrial chemicals	*Plasticizers* Di-butyl phthalate *Surfactants* Nonylphenol	• Domestic wastewater • Industrial activity
Pesticides	*Herbicides* Atrazine *Insecticides* Diazinon *Fungicides* Clotrimazol	• Agricultural runoff • Domestic wastewater

Personal care products (PCPs)

PCPs include a wide range of compounds such as antibacterial and antifungal agents (triclosan), polycyclic musks used as fragances (Galaxolide and Tonalide), UV filters (benzophenones, methoxycinnamates) and parabens among others. They are ubiquitously present in daily-use products such as toothpaste, soaps, cosmetics, etc and end up in the aquatic environment mainly through domestic wastewater.

Hormones

Natural sex hormones found in the environment include androgens (androstenedione, testosterone), oestrogens (17α- and 17β-estradiol, E2, estrone, E1, estriol, E3) and progesterone. In addition, synthetic hormones include 17α-ethinyl oestradiol (EE2), diethylstilbestrol and progestins commonly used in contraceptive pills, hormone replacement therapies or in the treatment of certain cancers. In addition, in animal farming, estrogens and progestogens are commonly administered as growth promoters (Andersson and Skakkebaek 1999) and, although with some restrictions regarding animals for human consumption, to manipulate sex populations in aquaculture (Piferrer 2001). Hormones are excreted and continuously released into the aquatic environment through direct discharge, WWTP effluent, and also through runoff of manure and sewage sludge used as amendment in agriculture (Kuster et al. 2004). The presence of both natural and synthetic steroidal hormones in water samples has been a common object of study (i.e.:(Behera et al. 2011, Cavallin et al. 2014, Huang et al. 2013).

Together with estrogenic or androgenic activity, water extracts can also show thyroid-like or thyroid disrupting activity (Jugan et al. 2009, Searcy et al. 2012). However to our knowledge, only one study has actually analytically assessed the occurrence of thyroid hormones (THs) (Svanfelt et al. 2010). This is of particular interest since, in addition to the normal excretion rate of unconjugated L-thyroxine (T4), estimated in 20 µg per person and per day (Svanfelt et al. 2010), the synthetic levothyroxine, chemically identical to the natural T4 and used in the treatment of hypothyroidism, is currently one of the most prescribed drugs worldwide (The Health and Social Care Information Centre, 2012; IMS Health, 2012).

In fact, due to the high production and consumption volumes, toxicity and low WWTP removal, levothyroxine has been recently included in several priority pollutant rankings (Howard and Muir 2011, Kools et al. 2008), highlighting the need to perform further environmental research (Dong et al. 2013).

Industrial chemicals

This category includes flame retardants (poly brominated diphenyl ethers, PBDEs) plasticizers (phthalates, benzoates) surfactants (octyl- and nonyl-phenol), perfluorinated sulfonates and carboxylic acids (perfluorooctane and perfluorooctanoic acid) among others. Bisphenol A (BPA), an organic compound commonly used in the synthesis of plastics and epoxy resins and present in food and beverage packages, can also be included in this group. Since they are present in a wide variety of products (detergents, plastic houseware, cloths, etc.) the main source of entrance into the aquatic environment is domestic wastewater. Other potential source is industrial wastewaters, for instance from industrial cleaning discharges (Luo et al. 2014).

Pesticides:

`Pesticide´ is the general term for a wide group of compounds and mixtures, both natural and synthetic, used to kill, reduce or repel different pests. Currently the EU Pesticide Database lists 528 pesticides and over 1300 active substances (http://ec.europa.eu/sanco_pesticides/public/?event=homepage#). They enter the aquatic environment mainly from agricultural runoff but also from domestic wastewater, and taking into account their domestic use, its input is highly dependent on a number of factors such as season, rainfall, etc.

1.2. Targets of micropollutants

As they comprise a great variety of substances, with different chemical structures and properties, micropollutants can target several organizational levels, although here we will focus on the following three: endocrine system, phase I and II biotransformation enzymes and immune system. It has to be noted that the following categories are non-exclusive, i.e.: the same substance can provoke detrimental effects in several systems at the same time. But, moreover, complex relationships exist between these systems as they will be later described.

Phase I and II biotransformation enzymes.

The main aim of the metabolism is to maintain cell homeostasis and it encompasses two opposite but complementary processes: anabolism (i.e.: synthesis of complex molecules from simple units, generally in reactions requiring energy) and catabolism (i.e.: degradation of complex molecules, what could lead to generation of the energy needed for the functioning of the organism or in some cases to the use of energy if

we refer to the degradation of non-desirable substances). When a xenobiotic enters the body, it needs to be transformed to facilitate its elimination. This biotransformation process typically involves two phases: metabolic activation in phase I followed by conjugation in phase II. The role of biotransformation enzymes in relation to pollutants is of high importance for several reasons. In the first place because they facilitate the excretion of xenobiotics, but also because in some cases, the produced metabolites are more toxic than the parent compounds. In addition, the overactivation of biotransformation processes by chemicals provokes important changes in the allocation of energy, resulting in a decrease of resources that can be dedicated to other essential functions such as growth, reproduction or maintenance of an appropriate activity in the immune system.

Here, we will focus on phase I enzymes, among which, those related with the cytochrome p450 (CYP) system are probably the most important.

Endocrine system

In recent years, some of the most commonly studied micropollutants are those targeting the endocrine system. They are known as “endocrine disruptors” (EDs) or “endocrine disrupting compounds” (EDCs) and have been defined as exogenous substances or mixtures that alter the function(s) of the endocrine system, causing adverse health effects on an intact organism, its progeny, or (sub) populations, even when present at very low concentrations (ng l−1) (WHO/IPCS, 2002). They include a wide variety of substances, both natural (i.e.: phytoestrogens, mycotoxins) (e.g.: Arukwe et al. 1999, Doerge and Sheehan 2002) and of anthropogenic origin (i.e.: Bisphenol A, parabens, phthalates) (e.g.: Morohoshi et al. 2005). A well known effect of this type of substances is, for instance, feminization of fish (Sumpter 1995, Matthiessen and Sumpter 1998).

Immune system

Another organizational level which can be affected by pollutants is the immune system. Referred to as the group of biological structures and processes whose ultimate function is to protect the organism against disease, the immune system plays a key role in an individual´s survival. In this sense, pollutants provoking changes in that ability of organisms to fight pathogens are of high ecotoxicological concern,

since they can potentially influence populations by increasing the susceptibility of individuals to disease (Bols et al. 2001).

In order to understand the mechanisms of action and the potential hazard towards living organisms, it is necessary to briefly describe the main features of these systems first. In the following sections, a brief description of each of the systems along with the common toxicity mechanisms will be done

2. METABOLISM OF XENOBIOTICS

In nature, organisms are continuously exposed to a great variety of potentially toxic compounds. In order to protect the body against such harmful substances, organisms have developed sophisticated detoxification systems, in which drug metabolizing enzymes (DMEs) constitute an essential part (Xu et al. 2005).

The liver is the main organ for biotransformation of xenobiotics in all vertebrates including fish. The metabolism of most lipophilic xenobiotics typically occurs in 2 phases and involves reactions of biotransformation (Phase I) and reactions of conjugation (Phase II). The objective of these two phases is to transform these lipophilic xenobiotics into a more polar metabolite and then to an even more polar conjugate to favor their excretion. In addition to these two phases, it is now accepted that two further steps, named Phase 0 and Phase III are also key players in the detoxification process. In Phase 0, the cellular entry of xenobiotics is modulated by a vast amount of transporters. In Phase III lipophilic xenobiotics or their metabolites, are pumped out of cells by specific transporter proteins (Fig. 2) (Xu et al. 2005, Szakacs et al. 2008).

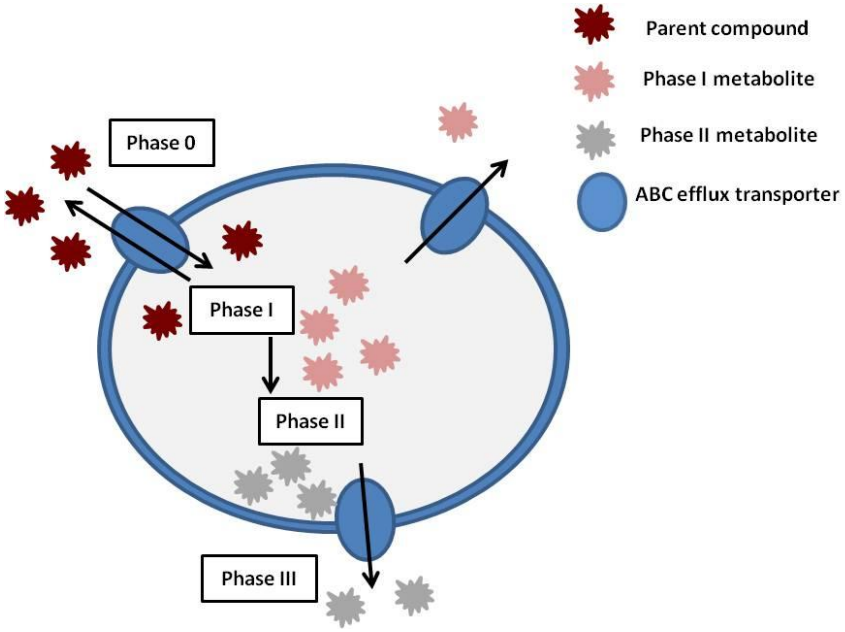


Fig. 1 Schematic representation of the possible cooperation of ATP-binding cassette (ABC) efflux transporters (phase 0 and phase III) and biotransformation enzymes (phase I and phase II) in cellular detoxification (Modified from Ferreira et al., 2014)

The phase I biotransformation involves reactions of oxidation, reduction, hydration and hydrolysis, and usually leads to the production of metabolites which contain hydroxyl groups. A wide variety of enzymes play a key role in this phase I (Table 2). Of these, the microsomal monooxygenases are the most versatile and are able to metabolize most lipophilic xenobiotics. Oxidations by this system depend on the activation of molecular O2 after it has been bound to a

hemoprotein, named CYP. Activation implies the transfer of electrons to the bound O2, which splits. One atom is used to oxidize the substrate (xenobiotic) and the other to form water. The reaction occurs in presence of NADPH, which acts as a donor of electrons (Walker et al. 2006):

$$NADPH2 + X + O2 \rightarrow XO + H20 + NADP$$

Cytochromes p450 exist in many different forms and they account for 70 to 80 percent of enzymes involved in drug metabolism. In humans there are approximately 60 CYP genes while across all the piscine species 137 genes, classified in 18 families, have been identified (Uno et al. 2012). It has to be noted that, although in the majority of cases, biotransformation leads to a loss of toxicity, in some occasions it results in the production of active metabolites, which are more toxic than the parent compounds. This occurs with some polycyclic aromatic hydrocarbons (PAHs) such as benzo(a)pyrene, whose oxidation leads to the formation of reactive metabolites (epoxides) that can bind to DNA (Rev. in Walker et al. 2006). The fact that some of these CYP dependent enzymes show a strong increase in their activity after exposure of organisms to particular groups of xenobiotics have made of them very good candidates to be used as biomarkers. Because of their value in biomonitoring and their interest in the context of the present thesis, CYP1 and CYP3 families, more specifically CYP1A and CYP3A will be further addressed in the following sections.

Table 2 Phase I enzymes (modified from Walker et al. 2006)

Name	Principal location	Cofactor	Substrate
Microsomal monooxigenases	Endoplasmic reticulum of many tissues (mainly liver of vertebrates, hepatopancreas, gut and fat body of invertebrates)	NADPH/NADH, O2	Most lipophilic xenobiotics (molecular weight<800)
Carboxyl esterases	Endoplasmic reticulum of many tissues, also cytosol and serum of vertebrates	None known	Lipophilic carboxyl esters
A esterases	Endoplasmic reticulum of certain cell types of vertebrates; mammalian serum.	Ca 2+	Organophosphate esters
Epoxide hydrolases	Endoplasmic reticulum of animal cells; some in cytosol	None known	Organic epoxides
Reductases	Endoplasmic reticulum and cytosol of several animal cells.	NADH/ NADPH	Organonitro-compounds, some organohalogens

Phase II (Table 3) enzymes, combine the metabolites generated in the previous phase with a polar endogenous group (e.g.: UDP-glucuronic acid or glutathione) to form conjugates. These phase II enzymes include glucuronyl transferases (UGTs) and sulfotransferases (SULTs).Most of these conjugates are negatively charged, have considerable water solubility and are readily excreted in bile and urine (Rev. in Walker et al. 2006). In vertebrates, the major pathway for conversion and inactivation of both endogenous (e.g.: steroid and thyroid hormones etc) and exogenous (e.g.: pharmaceuticals) compounds is glucuronidation. This pathway involves the transfer of an activated sugar group to the substrate, which increases the water solubility of the compound and in contrast to phase I, it does not require energy in the form of ATP or NADPH (Schlenk et al. 2008)

Table 3 Phase II enzymes (Walker et al. 2006)

Name	Principal location	Cofactor	Substrate
Glucuronyl transferases (UGTs)	Endoplasmic reticulum of many animal cells	UDP-glucuronic acid	Organic compounds with free OH groups; some organic compounds with free –SH or NH2
Sulfo-transferases (SULTs)	Cytosol of many animal cells	Phospho-adenine phosphor-sulfate	Many organic compounds with free OH groups
Glutathione-S-transferases (GSTs)	Mainly cytosol of many animal cells, some in endoplasmic reticulum	Reduced glutathione	Foreign electrophiles, including some organohalogens and organic epoxides.

Phase III transporters include for instance P-glycoprotein (P-gp) and multidrug resistance-associated protein (MRP). They transport substrates across membranes by using the energy from the hydrolysis of ATP and are therefore collectively known as ATP-binding cassette (ABC) transporters (Xu et al. 2005). In recent years, the presence of such transporters has been described for an increasing number of piscine species including rainbow trout, *Oncorhynchus mykiss* (Zaja et al. 2008, Fischer et al. 2011, Loncar et al. 2010).

2.1. CYP1A

Among the wide range of enzymes involved in the metabolism of xenobiotics, CYP1A, is probably the most relevant. It is induced in the presence of a broad variety of chemicals,but in particular dioxins and dioxin-like substances, and as a consequence it has been widely used as an *in vivo* biomarker of environmental exposure (Nebert et al. 2000) (see section 2.3) to such kind of compounds.

CYP1A induction is mediated via the aryl hydrocarbon receptor (AhR) (Stegeman et al. 1995), which is a ligand-dependent transcription factor belonging to the basic helix-loop-helix (bHLH)/PAS family that regulates the expression of a battery of genes (Nebert et al. 2000). AhR is located in the cell cytoplasm, in a complex with heat shock protein 90 (HSP90), the hepatitis B virus X-associated protein (XAP2) and p23. Upon ligand binding, it suffers a conformational change and is translocated into the nucleus, where it dissociates from the chaperone proteins and forms a heterodimer with the aryl hydrocarbon receptor nuclear translocator (ARNT). The heterodimer then, binds specific DNA sites, named dioxin or xenobiotic responsive elements (DRE or XRE, respectively), and initiates the transcription of target genes (Fig. 3). GThe list of genes directly regulated by the AhR, which continues to grow, includes some key Phase I and II metabolizing enzymes such as CYP1A1, CYP1A2 and CYP1B1 and glutathione S-transferase (GST)-Ya subunit among others (Beischlag et al. 2008, Ramadoss et al. 2005).

Typical AhR inducers are planar, polycyclic compounds including some polyaromatic hydrocarbons (PAHs), a number of dioxins, and some polychlorinated biphenyls (PCBs) (Denison and Nagy 2003). Among those inducers, the most potent agonist of AhR found up to now is the highly toxic and carcinogenic 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), which has a Kd in the picomolar range (Rowlands and Gustafsson 1997). Other prototypical ligands include for instance ß-naphthoflavone. However, increasing number of data indicates that AhR can also be activated by other structurally different non-planar compounds, including some drugs (Fernandez-Cruz et al. 2011), pesticides (Casado et al. 2006), fungicides (Navas et al. 2004) and even natural occurring substances (Behrens and Segner 2005). Interestingly, despite of this large and varied range of AhR-activating substances, a putative high-affinity endogenous ligand has yet to be identified and AhR remains, therefore, classified as an orphan receptor.

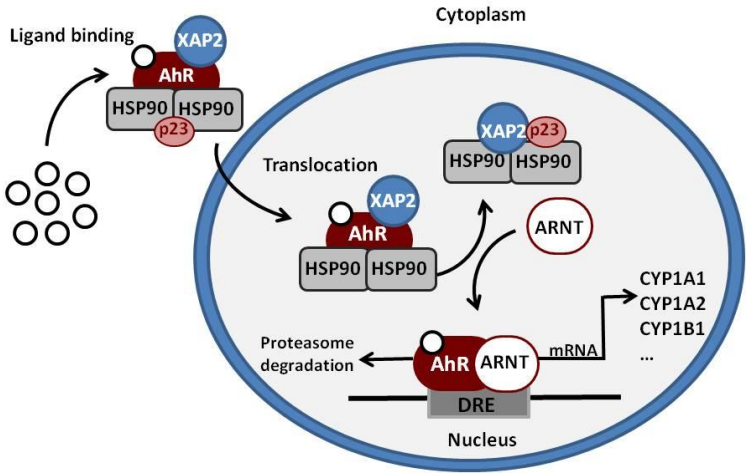


Fig. 2 Model of ligand-mediated translocation of the Ahr complex into the nucleus

In fish, two *ahr* genes have been identified: *ahr1* and *ahr2*. Although *ahr1* is thought to be the ortholog of the mammalian *ahr*, *ahr2* is the dominant form in most teleostean species (Hahn 2002). In addition, it has been found that *ahr1* is nearly undetectable in many tissues that show inducible *cyp1a* expression, suggesting that AhR2 is able to mediate the response (Powell et al. 2000) and justifying its selection in biomonitoring studies (Valdehita et al. 2012).

2.2 CYP3A

This cytochrome is also implicated in detoxification processes and pertains to the predominant CYP subfamily in both fish and human liver (Celander et al. 1996). Due to its wide range of substrates is probably one of the most important drug-metabolizing enzymes in vertebrates, contributing to the metabolism of approximately 50% of currently marketed drugs (Guengerich 1999). In mammals, its expression is regulated by the pregnane-X-receptor (PXR) (Kliewer 2003), an orphan nuclear receptor first described in 1997 and named after the observation that high concentrations of pregnanes (21-carbons steroids) activated the receptor (Kliewer et al. 1998). It is a highly promiscuous nuclear receptor activated by a wide range of structurally different compounds, including pharmaceuticals (e.g.: rifampicin, phenobarbital, hyperforin), steroids (e.g.: pregnenolone-16α-carbonitrile) as well as some environmental pollutants (e.g.: phthalic acid, nonylphenol). Upon ligand binding, human PXR dimerizes with the retinoid-X-receptor (RXR) and binds to specific DNA sites located in the CYP3A gene promoters (Kliewer et al. 1998), initiating transcription. Moreover, other genes involved in xenobiotic detoxification in the liver and intestine such as aldehyde dehydrogenases, glutathione-S-

transferases and P-glycoprotein (P-GP) transporter genes among others, appear also to be regulated by PXR (Kliewer et al. 1998). This promiscuity of the PXR, mostly attributed to its large (>1300Å^3) and flexible ligand-binding pocket, together with the activation of a complex network of genes involved in detoxification ensures an efficient clearance of numerous structurally diverse compounds (Kliewer 2003).

In comparison to mammalian models, much less is known about piscine PXRs and their regulation. In juvenile rainbow trout, PXR tissue expression was similar to that in mammals, with the liver and intestine exhibiting the highest levels (Wassmur et al. 2010). However, it seems to be regulated in a different manner and, similarly to other piscine PXRs, has proved highly unresponsive to prototypical mammalian ligands as for instance rifampicin (Wassmur et al. 2010). In fact, it appears that PXRs are activated in a species-specific manner. This particularity has been named “directed promiscuity”, meaning that PXRs from different species are activated by a diverse but precise array of compounds (Watkins et al. 2001). It has been suggested that the reason of such differences lies in the fact that the ligand binding domains (LBDs) of PXRs from different species exhibit important sequence divergence (Orans et al. 2005).

2.3. The use of CYP1A and CYP3A as biomarkers

Biomarkers have been defined as biochemical, physiological or histological changes that are indicative of exposure to or effects of xenobiotics at the organismal and suborganismal level (Mayer et al. 1992). In general the main advantage of biomarkers is that they serve as an early warning system which allow to elucidate whether pollutants are present at a concentration high enough to produce an effect. In this context, some of the most commonly used biomarkers (Schlenk et al. 2008, Celander 2011, Peakall and Walker 1994) are related to the CYPs.

Induction of CYP1A has been commonly used as a biomarker. This induction involves an increase in both cytochrome expression and in the associated enzymatic activities, meaning that its activation can be measured either at the protein (Sturve et al. 2006), gene expression (Kim et al. 2013) or enzymatic level. One of the enzyme activities dependent on CYP1A and commonly used for monitoring purposes is ethoxyresorufin-*O*-deethylase (EROD) (Jung et al. 2014), which has several advantages (Whyte et al. 2000). In the first place it is a relatively rapid and cost-effective method which provides a fingerprint of the exposure to AhR-activating compounds. In spite of not providing information on the identity and concentrations of contaminants in fish tissues, EROD demonstrates the cumulative impact of all inducing chemicals, taking into account

even those that are not detected by analytical means. Actually, this is one of the big advantages of the use of biomarkers and in particular of EROD activity, since it is possible to obtain information about the global toxic potency of the substance or sample analysed, and any additive, agonist or antagonistic effect will be reflected in the measured activity (on the contrary, analytical chemistry techniques do not yield an indication of the potency of induction or about possible interactions among substances present in a sample). However, it has to be noted that EROD activity may be influenced by a wide range of biotic and abiotic factors including water temperature and pH, age and reproductive stage among others, meaning that an understanding of these factors is essential when utilizing this biomarker (Whyte et al. 2000).

In contrast to CYP1A and EROD activity, extensively used as a biomarkers of environmental pollution (Valdehita et al. 2012, Jolly et al. 2012, Locatello et al. 2009), CYP3A and its associated enzymatic activity, benzyloxy-4-[trifluoromethyl]-coumarin-*O*-debenzyloxylase (BFCOD) have been applied for this purpose in only a few studies (Wassmur et al. 2010, Hasselberg et al. 2008, Hegelund et al. 2004). In fact while a quick search on Pubmed (http://www.ncbi.nlm.nih.gov/pubmed) with the terms “EROD” and “fish” yields over 1300 citations, demonstrating its widespread use as biomarker, the search “BFCOD” and “fish” renders only 10. A recent study by (Creusot et al. 2014) has demonstrated that typical AhR ligands (HAHs and PAHs, as well as imidazoles) induced BFCOD in piscine models both *in vitro* and *in vivo* while the AhR antagonist α-naphthoflavone was able to block the response. These controversial results suggest that further studies are needed to clarify if BFCOD activity is actually dependent on CYP3A, the usefulness of the use of CYP3A as a biomarker, the appropriate substrate to be used for the measurement of BFCOD and the receptors involved in the transcription process (Creusot et al. 2014).

3. THE ENDOCRINE SYSTEM.

The endocrine system of an organism is defined as the group of glands and tissues whose function is to produce and secrete hormones that exert particular regulatory functions in the target tissues. Hormones are chemical messengers able to modulate activities and functions of the organism at very low concentrations (in the range of pg/ml to µg/ml plasma). Once secreted they can reach target cells by direct communication between cells, through the intercellular spaces or, in most cases, through the bloodstream. Hormones are transported in the bloodstream either free or bound to transport proteins and exert their action on target cells in defined tissues and organs through interaction with specific receptors.

Currently, more than 130 hormones have been described in mammals (Norman and Litwak 1997), with most of them also found in teleost fish. This list is continuously being updated, as new hormones or new functions are constantly discovered. They regulate many crucial processes such as growth, metabolism, development, reproduction, etc. and according to their chemical structure, can be classified into the following groups:

- Peptidic: Most of the existing hormones are either oligopeptides or small polypeptides. They are synthesised in the ribosomes of the reticulum, generally as larger precursors named pro or preprohormones. These will suffer one (prohormones) or two (preprohormones) steps of proteolytic processing before the final hormone is formed. Subsequently, they will be packaged into secretory vesicles by the Golgi apparatus and secreted. Peptidic hormones include thyrotropin and gonadotropin releasing hormones (TRH and GnRH, respectively). In addition, peptides can associate to other peptides or compounds leading to complex structures, such as glycoproteins. This is for instance the case of thyrotropin (TSH) or gonadotropins (GTHs).

- Amino-acid based: They are all derived from the amino acid tyrosine and include thyroid hormones and catecholamines (epinephrine, norepinephrine and dopamine).

- Steroids: The totality of steroids (sexual hormones, mineralocorticoids, glucocorticoids, etc) are synthesized from cholesterol, which is the common precursor. This process, known as steroidogenesis, will be further addressed in the following sections (see section 3.2.1).

- Eicosanoids: They are formed from the oxygenation of 20-carbon fatty acids such as arachidonic acid. This category includes for instance the prostaglandins, leukotrienes, etc.

The chemical structure of the hormone will determine several characteristics including their mode of action. For instance, peptidic hormones are membrane insoluble and act via surface receptors and second messengers (cAMP, cGMP, etc). On the contrary, steroids and thyroid hormones, due to their low molecular weight and lipophilicity, are able to trespass membranes and interact with cytoplasmatic and nuclear receptors. The concrete mechanisms of actions by which two kinds of steroid hormones, estrogens, androgens, and one kind of the aminoacid-based hormones, thyroid hormones, exert their actions will be discussed later (see sections 3.2.3, 3.2.5 and 3.3.3, respectively).

The organs, tissues and glands of the endocrine system are usually functionally interconnected; we speak in these cases about “hormonal axes”. Although different axes exist, here we will focus on two of them: the hypothalamus-pituitary-thyroid (HPT) axis and the hypothalamus-pituitary-gonadal (HPG) axis (Table 4).

3.1 Organization of the Hypothalamus- Pituitary system in teleost fish.

The vertebrate neuroendocrine system consists of secretory neurons located in the hypothalamus and the hypophysis or pituitary gland, further separated into the adeno and neurohypophysis also named anterior and posterior pituitary, respectively. While the adenohypophysis contains the different cells synthetizing and secreting most pituitary hormones (e.g.: tyrotrophs, gonadotrophs etc), the neurohypophysis is formed by neurosecretory fibers originating from different parts of the brain and secreting various peptides into the vicinity of the pituitary cells. Despite some similarities and the functionality of both HPG and HPT axes being conserved across vertebrates, the anatomical organization of the hypothalamic-pituitary connection differs between mammals and fish. In mammals, neurons are connected with secretory cells by means of a portal blood system. Teleost fish however, lack such hypothalamus-pituitary portal connections. Instead, the hypophysiotropic neurons send their axonal projections directly into the anterior pituitary where they release the neurohormones, in the vicinity of their target cells (Zohar et al. 2010). A further difference is the arrangement of the hormone-producing cells (gonadotrophs, somatotrophs, thyrotrophs, etc) in the pituitary. While

in mammals they are arranged in a mosaic pattern, in fish each specific cell type is located in a different pituitary compartment (Maruska and Fernald 2011; Maruska 2011) (Fig.4).

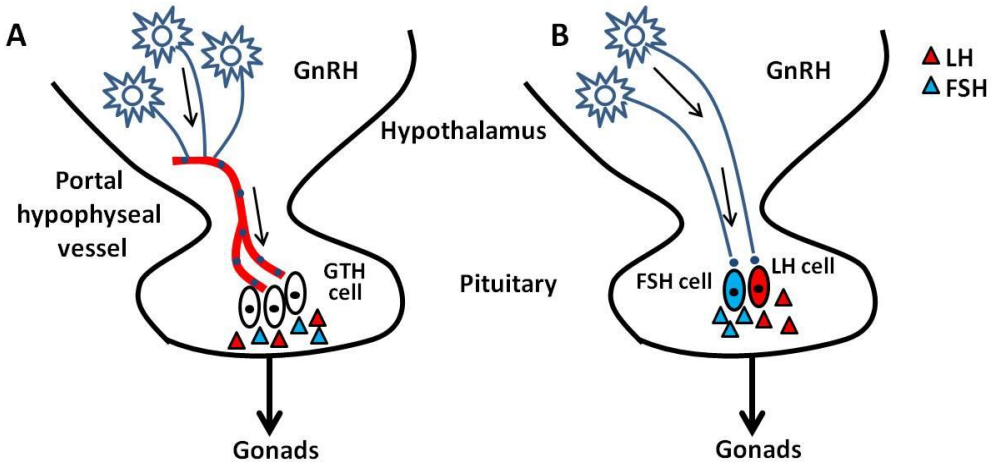


Fig. 3 Hypothalamus-pituitary system in mammals (A) and fish (B). Modified from Amano, 2010

Table 4 Organization of hypothalamus-pituitary-thyroid (HPT) and hypothalamus-pituitary-gonadal (HPG) axes.

Axis	Endocrine gland/origin	Hormone	Class	Target tissue	Function
HPT	Hypothalamus	Thyrotropin releasing hormone (TRH)	Peptidic	Pituitary	Regulates thyroid stimulating hormone (TSH) production
	Pituitary	Thyroid stimulating hormone or thyrotropin (TSH)	Glycoprotein	Thyroid follicles	Stimulates thyroid follicles to produce T4 and T3
	Thyroid follicles	L-thyroxine (T4) 3,3′,5-triiodo-L-thyronine (T3)	Iodinated amino acid	All tissues	Increase metabolic rate, regulategrowth and development
HPG	Hypothalamus	Gonadotropin releasing hormone (GnRH)	Peptidic	Pituitary	Regulates gonadotropins production (LH and FSH)
	Pituitary	Gonadotropins (GTHs) (Luteinizant, LH; Follicle Stimulating hormone, FSH)	Glycoprotein	Gonads	Controls reproductive development and function
	Gonads (ovaries and testes)	Progesterone	Steroid	Gonads, brain	Reproductive functions
		Estrogens (e.g. 17ß-estradiol)	Steroid	Brain, liver, kidney, bone…	Development and reproductive functions. Development of female sex characteristics
		Androgen (e.g. testosterone)	Steroid	Gonads, muscle, brain, bone…	Development and reproductive functions Development of male sex characteristics

3.2. Hypothalamus- Pituitary-Gonadal Axis: an overview.

In all vertebrates, reproduction is controlled by the well-conserved hypothalamus-pituitary- gonadal (HPG) axis. This is a dynamic system in which the different tissues are interconnected by neuronal (faster) and vascular (slower) linkages and the homeostasis is achieved by means of positive and negative feedbacks (Fig. 5) (Ankley and Johnson 2004).

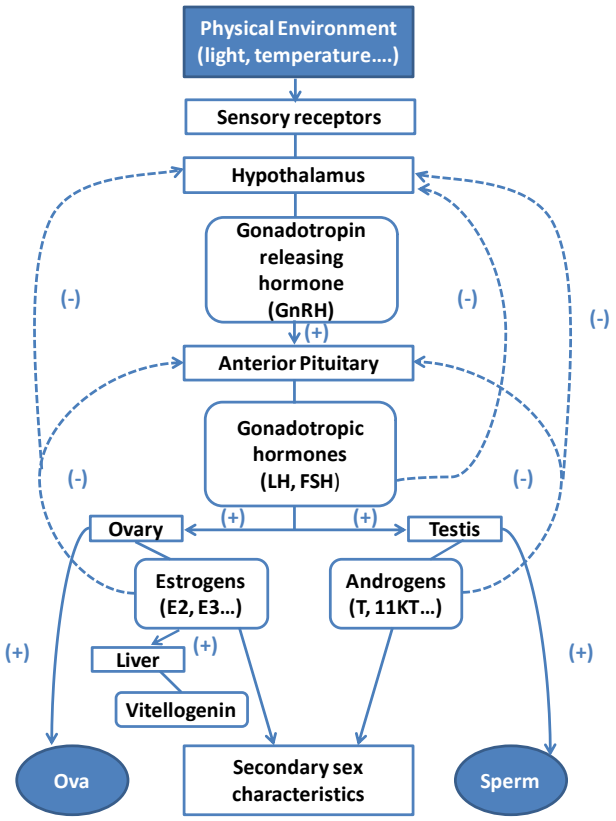


Fig. 4 Hypothalamus-pituitary-gonadal axis (modified from Moyle and Cech, 1988)

The summit of the HPG is located in the hypothalamus which produces and releases a peptidic hormone named gonadotropin releasing hormone (GnRH). The GnRH is a decapeptide hormone, well conserved among vertebrates, synthesized by neurosecretory cells in the hypothalamus and whose function is to control the release of the gonadotropins (GTHs). Since its discovery in the 70's (Burgus et al. 1971, Matsuo et al. 1971), more than 20 GnRH variants have been identified (Kah et al. 2007). All these variants, originally named after the first species in which they were found (e.g.: salmonGnRH, chickenGnRH), are now classified into three main branches of peptides, each of them produced by one of the following paralogous genes: *gnrh1, gnrh2* and *gnrh3*. Of these, *gnrh1* and *gnrh2,* widely expressed among vertebrates, give rise to GnRH1 and GnRH2 forms, respectively. On the contrary, the third group of peptides, encoded by *gnrh3* so far has only been found in teleost fish (Fernald and White 1999).

Hypophysiotropic GnRH is released to the pituitary, near the gonadotroph cells where it binds to the GnRH receptor (GNRHR). Upon binding to this G-protein coupled receptor, GnRH stimulates the synthesis and release of the two gonadotropins (GTHs): Follicle Stimulating hormone (FSH) and Luteinizing Hormone (LH). In addition to GnRH, other neurotransmitters and neuropeptides such as neuropeptide y (NPY) and Gamma-aminobutyric acid (GABA) have been shown to control the release of GTHs. Moreover the discovery of the central role of the kisspeptin and G-protein coupled receptor 54 (kiss1/gpcr54) system in the control of GnRH and GTHs release has meant a landmark in the understanding of the regulation of reproduction (Zohar et al. 2010).

GTHs (FSH and LH) are heterodimeric glycoprotein hormones, consisting of two subunits. They are formed by a common subunit (named α) and a specific one (named β), the later conferring the biological activity and specificity of the hormone. Once released into the blood, GTHs are transported to the gonads where they bind to their corresponding receptors, FSHR and LHR, that belong to a family of rhodopsin-like G-protein-coupled receptors. GTHs stimulate sperm production, oocyte growth and development and the synthesis and release of steroid hormones. While FSH primarily induces oogenesis and spermatogenesis, LH mainly causes final gamete maturation and induction of ovulation or sperm release (Ankley and Johnson 2004).

3.2.1 Steroidogenesis

Steroidogenesis is the process of biosynthesis of different steroid hormones including glucocorticoids, mineralocorticoids, progestins and sex hormones (Sanderson and van den Berg 2003). Both GTHs play a key role in the regulation of steroidogenesis although this phenomenon is highly dependent on LH action. The synthesis of steroids is initiated when the steroidogenic acute regulatory protein (StAR) transports cholesterol from the cytoplasm to the inner mitocrondrial membrane, where it is converted to the steroid precursor pregnenolone, in a reaction catalyzed by the CYP side-chain cleavage enzyme (P450scc or CYP11A). Pregnenolone is then subsequently transformed via different subpathways to androgens, estrogens etc., in reactions accomplished by a variety of enzymes including several CYPs, hydroxysteroid dehydrogenases (HSD) and steroid reductases (Miller 1988)(Fig. 6) (Payne and Hales 2004). In males, synthesis of testosterone (T), the prototypical androgen, occurs in Leydig cells. Thereafter it can be converted into the most potent endogenous androgen dihydrotestosterone (DHT) by steroid 5α-reductase or into E2 by the aromatase CYP19. T may be transformed to 11β-hydroxytestosterone (OHT) by the action of the CYP11B. OHT is subsequently transformed into

11-ketotestosterone (11-KT), the main androgen in fish, by action of 11β-HSD. In females, T is synthesized in the theca cells of the follicle, and thereafter released to the granulosa cells, where it is converted to E2, the major female sex hormone and responsible for stimulating hepatic vitellogenesis. In teleost fish, two isoforms of the aromatase have been described. They are encoded by distinct genes and are differentially expressed among tissues, with CYP19A mostly expressed in gonads and CYP19B in brain (Cheshenko et al. 2008).

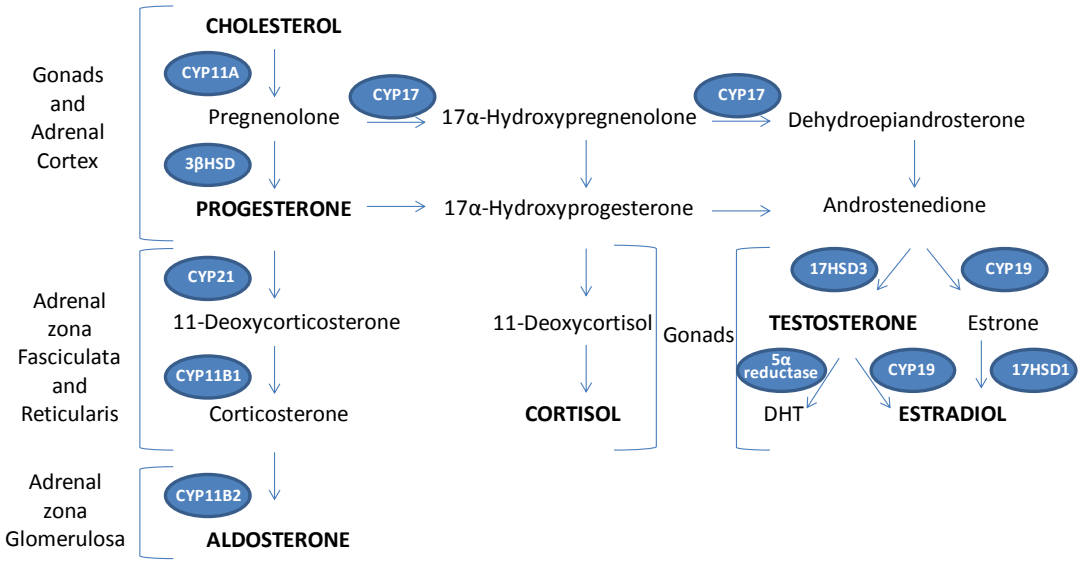


Fig. 5 Biosynthesis of steroid hormones in adrenal glands and gonads (Modified from Payne and Hales, 2004)

3.2.2. Steroid metabolism

The liver is the main site of peripheral inactivation and catabolism of all steroids including estrogens and androgens. Given their lipophilic nature, the majority are excreted in the urine as inactive conjugates (sulfates or glucuronides) that are formed in reactions catalyzed by SULTs or UGTs, respectively. Although some estrogens and androgens are also excreted unconjugated, the presence of free (biologically active) forms in the environment has been mainly attributed to the biotransformation activity of bacteria (Panter et al. 1999, Shore and Shemesh 2003). Given the nature of our work, we will focus mainly on estrogens.

3.2.3. Genomic mechanisms of estrogens action.

Estrogens such as E1, E2 and E3 are involved in a great variety of physiological functions including oogenesis (Miura et al. 2007), vitellogenesis (Nagahama 1994), and regulation of GTHs secretion (Mateos et al. 2002) among others. They exert their action through binding to

intracellular estrogen receptors (ERs), which belong to the nuclear receptor superfamily and act as ligand-dependent transcription factors. In most piscine species, three ERs, ERα, ERβ1 and ERβ2, with tissue specific expression and different binding affinities have been identified, being the product of distinct genes. In rainbow trout however, an additional form, named ERα2 and presumably generated by a recent gene duplication, has been described (Nagler et al. 2007).

In the absence of ligand, ERs are mainly found as monomers complexed with inhibitory heat shock proteins (Nelson and Habibi 2013). Upon ligand binding, the heat shock protein complex is dissociated and the receptor is translocated into the nucleus, where it forms a dimer. Although ERs are thought to function primarily as homodimers, ERα-ERβ dimers have also been found (Cowley et al. 1997). Then, the dimer interacts with specific DNA regions, the estrogen response elements (ERE) and initiates transcription of target genes.

3.2.4. Non-genomic mechanisms of estrogen action.

In addition to this mechanism of action (known as classic or genomic), increasing evidence suggests that estrogens can also activate receptors on the cell surface initiating rapid biological responses. They are the so-called non-classical pathways (Thomas 2012). On the contrary to the classic genomic mechanism of action, which involves new mRNA and protein synthesis and typically occurs over a time scale of hours, the non-classical actions may occur within minutes. Although the exact mechanisms are diverse and in some cases still under debate it is thought that at least part of these actions are mediated by an orphan G protein-coupled receptor named GPCR30 (Thomas 2012).

3.2.5. Androgens and AR-signaling

Androgens, the male sexual hormones, are key players in male sexual differentiation and development. In teleost fish, the major circulating androgens are testosterone (T), 11-ketostestosterone (11-KT), 11 β-hydroxytestosterone (OHT) and 11B-hydroxyandrostenedione (OHA) (Knapp and Carlisle 2011). Both T and OHA are derived from androstenedione by action of 17β-HSD and CYP11B1, respectively. OHT in turn is synthetized from T by CYP11B1 and subsequently transformed into 11-KT by action of 11 β-HSD. In fish, T is present in the blood of females at similar (or even higher) concentrations than in males, therefore suggesting that is not the principal androgen (Campbell 1980). This role of principal androgen has been attributed to 11-KT, found only in males and responsible for the development of secondary sexual characters

and sexual behavior (Godwin 2010). Androgens exert their action through binding to the androgen receptor (AR), which is also a ligand-inducible transcription factor. Upon ligand binding, AR suffers a conformational change which leads to nuclear translocation, increased phosphorylation and homodimer formation. The AR dimer binds to androgen-response elements (ARE) in the regulatory regions of target genes and regulates its transcription (Li and Al-Azzawi 2009). In teleost fish the number of androgen receptors varies depending on species. In rainbow trout, two receptors have been identified: ARα and ARβ (Takeo and Yamashita 1999).

3.3 Hypothalamus- Pituitary-Thyroid Axis: an overview

Thyroid hormones (THs) play critical roles in growth, metabolism, and development in all vertebrates, including fish (Power et al. 2001). Their production for the maintenance of stable levels is regulated by the hypothalamus-pituitary-thyroid axis (HPT) through positive and negative feedbacks (Fig. 7)

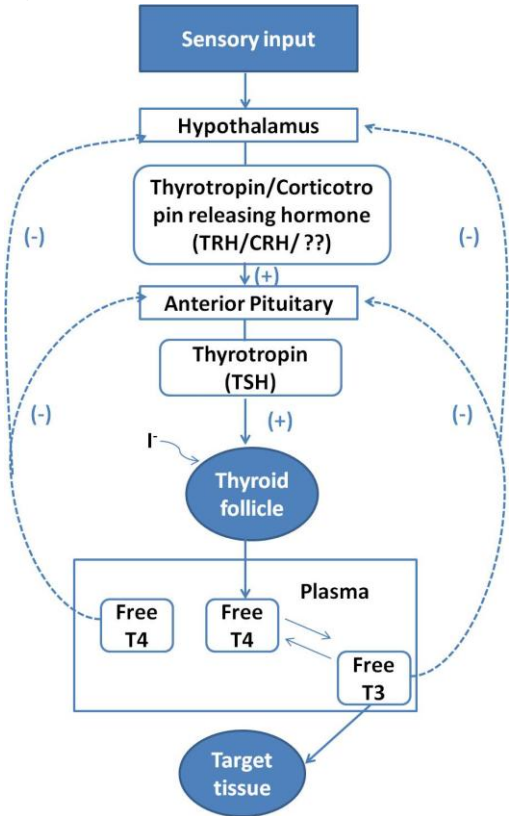


Fig. 6 Hypothalamus-pituitary-thyroid axis. Modified from (Eales et al. 1999).

In mammals, thyrotropin-releasing hormone (TRH), a peptidic hormone produced in the hypothalamus and highly conserved among vertebrates, stimulates the release of thyrotropin or thyroid stimulating hormone (TSH) from thyrotropes in the anterior pituitary. In teleost fish, however, this fact is less well established, and instead, corticotrophin-releasing hormone (CRH)

appears to play an important role as a TSH-releasing factor (De Groef et al. 2006). TSH is a dimeric glycoprotein hormone that shares the common alpha subunit with LH and FSH. Upon ligand binding to its receptor (TSHR), a member of the G protein-coupled receptor superfamily (Kumar and Trant 2001), it stimulates the secretion of L-thyroxine (T4) by the thyroid. In contrast to mammals, where thyroid exists as an encapsulated gland located in the throat, piscine thyroid presents a high heterogeneity in terms of form and location. For most teleostean fish, including rainbow trout, it consists of non-encapsulated follicles scattered along the subpharyngeal region surrounding the ventral aorta (Gudernatsch 1911). However, the existence of a compact gland or the localization of the follicles outside the typical subpharyngeal region has been described for some species (Baker 1958, Hamlin 2014). The follicles, considered the functional unit of the thyroid system, are formed by epithelial cells (thyrocytes) enclosing an extracellular space in which they secrete a glycoprotein called thyroglobulin. Then, inorganic iodide is actively scavenged from the blood by the sodium/iodide symporter (NIS) and incorporated into the thyroglobulin. After successive oxidization by the thyroid peroxidase (TPO), T4 is produced from the thyroglobulin protein (Blanton and Specker 2007) and secreted into the blood where T4 can be either free or bound to transport proteins. In teleost fish, around 99% of T4 in the blood is bound to transport proteins, either albumin or transthyretin, (TTR), meaning that only 1% circulates free and is readily accessible to target cells.

Once secreted into the blood, T4 enters target cells, located in peripheral tissues (i.e.: liver, kidney, etc.) where it undergoes monodeiodination and it is converted into the more biologically active 3,3′,5-triiodo-L-thyronine (T3) (Fig.8).

The reactions by which one hormone is converted into another (either activated or deactivated) are catalyzed by deiodinase enzymes. These are integral membrane selenoproteins catalyzing outer or inner ring deiodination (ORD and IRD, respectively) and thus regulating TH concentrations in blood. Up to date, three deiodinases have been characterized: Type I (D1), type II (D2) and type III (D3), which mainly differ in the type of reaction catalyzed. While D2 only catalyzes ORD, D3 only catalyzes IRD. In general, D2 catalyzes the conversion of T4 into the active T3 while D3 inactivates T3 and, to a lesser extent, prevents the activation of T4 by generating the inactive reverse T3 (rT3).

D1 on the contrary is non selective and able to catalyze both types of reactions (ORD and IRD) but showing a higher efficiency for ORD of rT3 (Darras and Van Herck 2012) (Fig7). T2 is an inactive product common to both pathways. The maintenance of stable levels of THs is tightly controlled by means of complex feedbacks and compensatory mechanisms. On the one hand, circulating T4 and, to a lesser extent T3, provide a negative feedback to the hypothalamus and pituitary, meaning that when the level is lowered, both TRH and TSH are activated. But, moreover, deiodinases activity is also regulated by feedbacks, providing a secondary via of THs homeostasis.

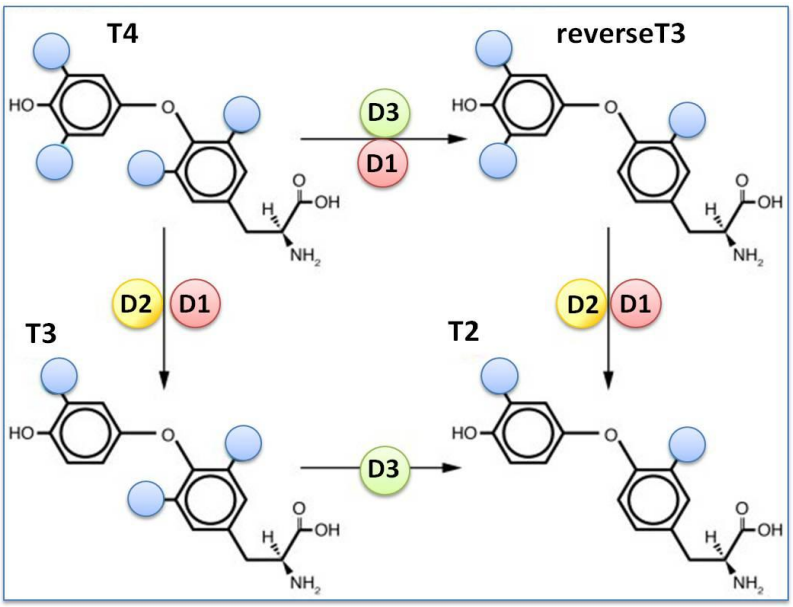


Fig. 7 Basic reactions of deiodination. Deiodinases may remove iodine moieties (blue spheres) from the phenolic (outer) or tyrosil (inner) rings of the iodothyronines. Deiodinases may activate T4 to T3 (via D1 or D2) or prevent its activation by converting it to reverse T3 (via D1 or D3). T2 is an inactive product common to both pathways (Modified from Bianco and Kim 2006).

3.3.1. Thyroid hormone clearance.

In all vertebrates, THs are excreted via bile through feces or urine. Conjugates of both T4 and T3 occur principally in the liver by means of sulfation or glucuronidation. But, in addition, piscine T4, and to a lesser extent T3, can cross the gill epithelia, which represents a secondary via of excretion (Zoeller 2005, Zoeller et al. 2007).

3.3.2. Genomic mechanisms of thyroid hormone action.

Classically, THs exert their action mainly through direct binding to the thyroid hormone receptors (THRs) (I.e.: Chin and Yen, 1997), which similarly to ERs, pertain to the superfamily of nuclear receptors. These are ligand-dependent transcription factors able to interact with specific DNA regions named TRE (thyroid hormone response element) and to regulate in turn the expression of a wide range of genes. To do so, they recruit auxiliary proteins, corepressors or coactivators, which act by modifying the chromatin or by interacting with the transcription mechanisms resulting, respectively, in the repression or activation of the target genes (Fig. 9).

Usually, THRs form heterodimers with retinoid X receptors (RXRs) although other receptors such as retinoic acid receptors (RARs) have been also found to be robust partners (Lee & Privalski, 2005). Typically, in the absence of hormone, THRs recruit corepressors, such as nuclear receptor co-repressor (NcoR) (Horlein et al. 1995)or silencing mediator for RAR and THR (SMRT) (Chen and Evans 1995) and repress gene expression. Upon ligand binding, they release the corepressors, interact with coactivators and initiate target gene expression.

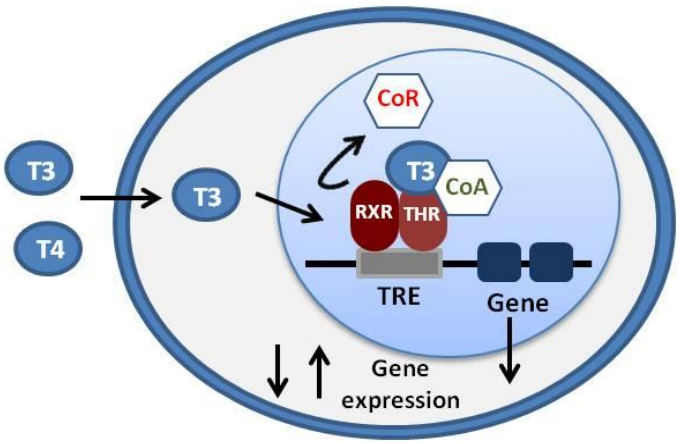


Fig. 8 Mechanism of thyroid hormone receptor action

Up to date, two main subtypes of THRs, THRA and THRB have been described in vertebrates. In fish, they are the product of at least two (e.g.: rainbow trout; Jones et al. 2002, Marchand et al. 2001) or three (e.g.: Japanese flounder, *Olive flounder*; Yamano et al. 1994, Yamano and Inui 1995) distinct genes which can in turn, generate several isoforms by alternate splicing). Although both T3 and T4 are able to bind THR, the former is known to have a higher binding affinity than the latter (Yen et al. 2006).

3.3.3. Non-Genomic mechanisms of thyroid hormone action.

Although initially it was considered that TH signaling is exclusively THR-mediated, it is nowadays accepted that some TH actions occur independently of intranuclear binding of the hormone. They are the so-called non-genomic or non-nuclear pathways, and in some cases they occur too quickly (seconds to minutes) for being initiated after T3 binding to nuclear receptors, (Rev. in Davis and Davis 2003). Even though different mechanisms of non-genomic actions have been described (Rev. in Davis et al. 2008), the recent finding in mammals of a cell surface receptor on a structural protein of the plasma membrane (integrin αVβ3) (Bergh et al. 2005) appears to be the source of at least part of them. Both T3, rT3 and T4, the latter with higher affinity, are able to bind this integrin, activating a mitogen –activated protein kinase (MAPK) signal transduction cascade.

3.4. Endocrine disruption.

In the 1990's a group of scientists proposed, based on a gross body of evidence, that certain chemicals widespread in the environment were affecting the endocrine systems of humans and wildlife (Colborn and Clement 1992, Colborn et al. 1993). They are the so-called endocrine disrupting compounds (EDCs), defined by the U.S. Environmental Protection Agency (U.S.EPA) as any "exogenous agent that interferes with synthesis, secretion, transport, metabolism, binding, action, or elimination of natural blood-borne hormones that are present in the body and are responsible for homeostasis, reproduction, and developmental processes" (EPA, 2012). As a consequence of the worldwide increasing concern, EDCs have been the subject of research of thousands of studies and reports (World Health Organization, United Nations Environment Programme WHO/UNEP, 2013) and currently, more than 900 substances are suspected of acting as EDCs (Institute for Environment and Health, IEH, 2005). Although this is a highly heterogeneous group which includes man-made chemicals (PCBs, dioxins, phthalates, Bisphenol A), pesticides (prochloraz, dichlorodiphenyltrichloroethane, DDT), pharmaceuticals (EE2, diethylstilbestrol) and natural compounds (genistein, coumestrol), in general EDCs share some common features: lipophilicity, persistence and bioaccumulation potential (Argemi et al. 2005). These characteristics have favored their worldwide dispersion and biomagnification in organisms situated on top of the trophic chain such as marine mammals or birds of prey.

Given the delicate equilibrium of hormonal systems, in which the timing and the concentration of the hormone is crucial, exposure to EDCs, even at low concentrations, may

result in a plethora of adverse effects. These include, for instance, diminished fertility and reproduction (e.g.: Gerbron et al. 2014), eggshell thinning (e.g.:Odsjo and Sondell 2014), altered sex differentiation (e.g.:Baumann et al. 2014) and alteration of the immune response (e.g.:Cabas et al. 2012) among others. In humans, the prevalence increase in several types of cancer (e.g.: breast or prostate) or diseases such as diabetes or autoimmune disorders have also been linked to the exposure to these substances (WHO/UNEP, 2013). Because of the key role of endocrine system in development, it is worthy to note that the effects of EDCs are highly dependent on the life stage when the exposure occurs, with embryos or young organisms being especially sensitive. Therefore, given this particular vulnerability in the early life stages, the maternal transfer of EDCs to the developing embryo and the consequential transgenerational effects is of special concern. For instance, in utero exposure to PCBs, known to disrupt TH-mediated brain development (Rev. in Gilbert et al. 2012) has been linked to neurodevelopmental impairment and poorer cognitive abilities in children (Jacobson and Jacobson 1996, Patandin et al. 1999). On the other hand, a subtoxic dose of lindane (25 mg/kg) administered to female rats during pregnancy, had no effect on the mothers but provoked reproduction-related adverse effects (i.e.: reduced sperm concentration and count etc.) in the male offspring (Di Consiglio et al. 2009).

3.4.1 Mechanisms of action of EDCs

The heterogeneity of EDCs, with a variety of chemical structures and properties, together with the complexity of the endocrine system, difficult the task of fully understanding the exact mechanisms by which endocrine disruption occurs. In fact, for a long time, it was thought that most effects were mediated by members of the NRs superfamily, giving to ER and possible estrogenic effects of chemicals a preponderant role. However, it is nowadays recognized that endocrine disruption is a more complicated phenomena than initially thought knowing that a wide variety of receptors and enzyme activities can be involved. Possible mechanisms of endocrine disruption are diverse (Argemi et al. 2005, Arukwe 2001, Howdeshell 2002):

- EDCs can mimic the biological activity of a hormone into the target cell by activating the corresponding cellular receptor (agonism), provoking abnormal cell activity and causing additive or even synergic effects. For instance, some phthalates and pesticides such as DDT are estrogenic because they act as agonists of the ER (Takeuchi et al. 2005) (Fig. 10A).

- They can reduce or block the normal activity of the endogenous hormone (antagonism). Substances with this mode of action include, for instance, dioxins and PCBs, which by a variety of mechanisms are able to impair the activity of the ER (Navas and Segner 2008) (Fig 10B).
- They can interfere with the synthesis or metabolism of hormones, altering the balance of circulating hormones. This is the case of lindane, which modulates the activity of several CYP members involved in the metabolism of steroids (Di Consiglio et al. 2009) or some brominated flame retardants, able to inhibit thyroid hormone SULT activity (Butt and Stapleton 2013). Another well known example is that of the inhibition of the thyroid peroxidase (TPO), necessary to form T4. A number of chemicals (i.e.: 6-propyl-2-thiouracil, PTU) as well as some isoflavones (i.e.: genistein, coumestrol) are known to block the activity of this enzyme, decreasing circulating levels of THs, and triggering an increase in TSH (Fig. 10C).
- They can bind to transport proteins in the blood, which may result in decreased ability of the hormone to reach its target tissue but may also facilitate the transport of the bound chemicals across the organism. For instance, the synthetic estrogen ethynylestradiol (EE2) has been shown to bind the sex hormone binding globulin (SHBG) with higher affinity than the endogenous hormones. This is of particular relevance, since it has been shown that fish are able to rapidly sequester trace concentrations of EE2 from water possibly through binding to SHBG in the gills (Miguel-Queralt and Hammond 2008). Other examples include some brominated flame retardants such as pentabromophenol (PBP) and tetrabromobisphenol A (TBBPA), which bind to transthyretin (TTR, the serum protein carrier of T4) with an affinity between 7 and 10 fold higher than that of T4 (Meerts et al. 2000) (Fig. 10D).

Despite this large and varied amount of mechanisms of action of EDCs, briefly described, and although any hormonal system of the organism may be affected, here we will focus on the processes mediated by three nuclear receptors through different molecular mechanisms:

- ER-mediated
- THR-mediated
- AhR-mediated

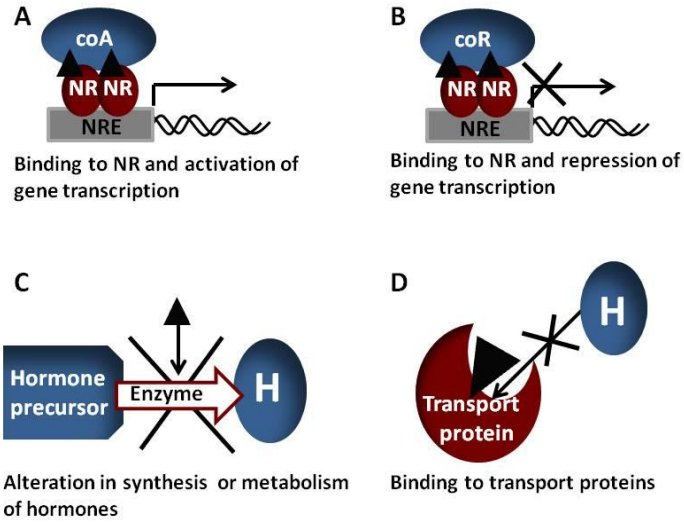


Fig. 9 General mechanisms of endocrine disruption. Endocrine disruptor is represented with a dark triangle.

3.4.1.1. ER-mediated endocrine disruption.

Among EDCs, the most studied are probably those directly interfering with the estrogen signaling pathway mediated by the ERs, which as transcription factors, regulate gene expression in response to estrogen exposure (usually in the pico-to femto-molar concentration range) (see section 3.2.3). ERs are considered as relatively promiscuous receptors, with large binding pockets (450Å), and therefore able to embrace a broad variety of non-steroidal substances. These, collectively known as xenoestrogens, include a wide array of natural (phyto- or myco-estrogens) and industrial compounds (pesticides, UV-filters, plastizicers, etc) that bind to ERs, exerting agonistic or antagonistic responses in a subtype-specific manner (Delfosse et al. 2014). This means that each environmental ligand binds ERα or ERβ with different affinity (Dang et al. 2011, Meerts et al. 2001). In fact, this difference in EDCs binding affinities between ERs subtypes

is bigger than the difference among interspecies ERs (Dang 2010), being the same compound able to exert opposite effects (either agonism of antagonism) depending on the receptor subtype. These differences may be related with the binding pockets. Indeed, it has been shown that the human ERα ligand binding pocket is bigger than that of the human ERβ, suggesting that the former is less exclusive than the latter. In addition, both receptors have an influence on each other at the transcriptional level, with ERβ acting as a dominant negative regulator for estrogen signaling (Dang et al. 2011). On the other hand, some substances exhibit more complicated modes of action. This is the case of selective estrogen receptor modulators (SERMs), whose activity depends on several factors such as the cell type or receptor subtype. For instance, raloxifene or tamoxifen act in a tissue-selective manner, e.g.: as agonists in bone but as antagonists in breast (Paech et al. 1997, Thiebaud and Secrest 2001).

Because of the roles of estrogens in normal physiology of fish, activation of the estrogen receptors by exogenous compounds may result in a wide variety of adverse effects, mostly related with development or reproduction (Rev. in e.g.: Milnes et al. 2006, Söffker and Tyler 2012). For instance, estrogenic substances, can skew the sex ratio towards females (Jin et al. 2012, Nimrod and Benson 1998), reduce spermatogenesis (Kiparissis et al. 2003, Kwak et al. 2001), provoke atrophy of the gonads and demasculinization (Lu et al. 2010, Gimeno et al. 1998), delay maturation of ovaries (Kiparissis et al. 2003), reduce fecundity (Nash et al. 2004) and provoke induction of vitellogenin in males (Lu et al. 2010, Song et al. 2014), the latter widely recognized and used as a sensitive biomarker of estrogen exposure. In addition, recent data has pinpointed to the notion that the immune system may be a target for EDCs (Rev.in Casanova-Nakayama et al. 2011, Milla et al. 2011).

Conversely, exposure to antagonists of the ER such as tamoxifen induces skewed ratio towards males (Singh et al. 2012) and masculinization of genetic females (Kitano et al. 2007).

Due to its high reproducibility, cost-effectiveness and easiness to establish high-throughput approaches, the use of *in vitro* assays is the tool commonly chosen to assess the potential (anti) estrogenic activity of a given compound or mixture. Among them, the induction of vitellogenin in hepatocytes (Navas and Segner 2006) or the use of transactivation assays using stably transfected cell lines have been proved very useful in a wide variety of matrixes including sediments (Tollefsen et al. 2008), water samples (Rao et al. 2013), and manure (Valdehita et al. 2014)among others.

3.4.1.2. THR-mediated endocrine disruption.

In recent years the number of studies reporting the adverse effects of a wide variety of chemicals on thyroid function has increased exponentially. Because thyroid axis is a complex system controlled by feedbacks and EDCs can disrupt nearly every step in the production and metabolism of THs (Howdeshell 2002), the understanding of the mechanisms of action behind thyroid disruption has proven to be very difficult (Jugan et al. 2010).

Based on the fact that generally pollutants bind THRs with relatively low affinity (Cheek et al. 1999) it was initially speculated that thyroid disruption was not likely to occur at this level (Howdeshell 2002, De Vito et al. 1999). However, recent data has provided new insights on THR regulation, being now increasingly admitted that chemicals can exert effects both on THRs and on their transcriptional activity (Rev. in Zoeller 2005, Jugan et al. 2010). In this context, transactivation (e.g.: Jugan et al. 2007, Freitas et al. 2011) and proliferation assays, the latter assessing hormone-dependent cellular growth (e.g.: T-screen, Gutleb et al. 2005, Ghisari and Bonefeld-Jorgensen 2005) are regarded as useful tools to screen both chemicals and environmental samples for their agonistic and antagonistic activities. By using such assays, the ability of many chemicals to induce or inhibit THR-mediated transactivation has been demonstrated. For instance, (Iwasaki et al. 2002) showed that a low dose of a specific hydroxylated PCB (OH-PCB) was able to suppress THR-mediated transcriptional activity in several cell lines. Moreover, using a model of neural progenitor cells, (Fritsche et al. 2005) demonstrated the ability of PCB-118 to mimic T3-induced differentiation.

A few environmental pollutants such as the flame retardant tetrabromobisphenol A (TBBPA) and some hydroxylated PBDEs and PCBs are able to bind THRs, as shown by competitive binding assays (Marsh et al. 1998, Kitamura et al. 2008, Kitamura et al. 2005a). Other examples of chemicals shown to interfere with T3 binding to THR include, for instance, BPA (Moriyama et al. 2002).

As relatively few chemicals exhibit affinity for THRs, other mechanisms, apart from direct ligand binding to the receptor, may be involved in the disruption of THR dependent transcription (Jugan et al. 2010). For instance, it has been suggested that some chemicals may mimic T3 activity without binding to THR but by interfering with the recruitment of coactivators and correpressors. This is the case of BPA and tributyltin chloride (TBTCl) which reduced T3-mediated gene transcription both by inhibiting T3 binding to THR and by recruiting the nuclear correpressor NcoR (Moriyama et al. 2002, Sharan et al. 2014). Other mechanisms include for instance, dissociation of THR:RXR from the TRE as provoked by some PCBs (Miyazaki et al. 2004).

In addition, the situation in some cases is even more complex as the same compound may disrupt thyroid hormonal activity via two or more different mechanisms. Is the case for instance of TBBPA, able to bind *in vitro* TTR (Meerts et al. 2000) and to act as an antagonist to the THR (Kitamura et al. 2005b).

Similarly to ERs, it has been suggested that chemicals may interact with THRs in an isoform-dependent manner. For instance, in a study performed with several PBDEs, a T3-like PBDE-OH selectively increased T3-mediated activation of THRA but had no effect on THRB while PBDE28 provoked the opposite effect (Schriks et al. 2007). In addition some chemicals may act as agonists or antagonists depending on the THR subtype. This is the case of the pharmaceutical compound desethylamiodarone, which acts as a noncompetitive inhibitor of T3 binding to THRB, but a competitive inhibitor of T3 binding to THRA (Bakker et al. 1994, van Beeren et al. 1995).

The HPT axis shows very complex regulatory feedback mechanisms and EDCs could impact thyroid homeostasis at different levels. As a consequence, it is very difficult to determine the actual mechanisms underlying the effects observed on the thyroid system. For instance, chemicals acting directly on THRs may produce variable and unpredictable effects on hormone levels (Zoeller 2005), which can in turn trigger compensatory mechanisms, and therefore affect a wide variety of biological processes. Taking all this into account, it is worth to outline some of the commonly found effects of thyroid disruption, independently of the mode or modes of action involved.

Provided the key role of THs in brain development, this has been one of the most studied effects of thyroid disrupting compounds with both *in vitro* (e.g.: Ibhazehiebo et al. 2011) and *in vivo* models (e.g.: Gauger et al. 2004). For instance, PBDE-209 inhibited TH-induced dendrite development of Purkinje cells, suggesting that by interfering with THR-mediated gene expression it may disrupt normal brain development (Ibhazehiebo et al. 2011). In another experiment performed with rats, it was shown that while mothers exposed to a mixture of PCBs exhibited decreased circulating THs, the brains from their fetuses presented increased expression of genes positively regulated by THs. This is therefore consistent with the hypothesis of a direct activation of THRs by PCBs in the fetal brain (Gauger et al. 2004). In amphibians, highly dependent on the proper functioning of HPT axis for normal development, some of the most commonly studied effects are related to metamorphosis. For instance, BPA and related compounds (TBBPA, TCBPA and TMBPA) suppressed T3-induced tail regression in Japanese wrinkled frog (*Rana rugosa*) and inhibited spontaneous metamorphosis in Western clawed frog (*Silurana tropicalis*). Further experiments with transgenic African clawed frog (*Xenopus laevis)*

tadpoles, revealed that those compounds acted by preventing binding of T3 to THR and therefore suppressing THR-mediated gene expression (Goto et al. 2006). Although most studies on fish up to date have measured thyroid disruption as changes on circulating THs and on expression of TH-responsive genes (e.g.: Guo and Zhou 2013, Shi et al. 2009, Yu et al. 2014, Li et al. 2014a, b), some authors have also assessed other endpoints. For instance, some evidence indicates that TH may have a role in gonadal sex differentiation, although in a species-specific manner. For instance, hypothyroid conditions provokes male-biased cohorts in three-spined stickleback (*Gasterosteus aculeatus*) (Bernhardt et al. 2006) and female-biased cohorts in zebrafish (*Danio rerio*) (Mukhi et al. 2007).

3.4.1.3. Aryl hydrocarbon receptor mediated endocrine disruption.

In addition to the mechanisms previously mentioned, endocrine disrupting effects can also be mediated through the AhR (Frye et al. 2012). AhR is an orphan receptor which acts as a ligand-dependent transcription factor, modulating the transcription of several genes, including *cyp1a1* and *cyp1a2*, among others (see section 2.1). AhR is typically (but not exclusively) activated in the presence of planar, polycyclic and aromatic compounds such as TCDD and planar PCBs, among others.

Activation of this receptor has been commonly associated with antiestrogenic effects both *in vivo* (Navas et al. 2005) and *in vitro* (Navas and Segner 2000). Antiestrogenicity has been defined as "any inhibition or reduction of estrogen induced processes due to interferences with the normal functioning of the estrogen receptor pathway" (Navas and Segner 2008) and, although not completely understood, several mechanisms have been proposed (Navas and Segner 2008, Swedenborg et al. 2009):

- ER and AhR can compete for common co-activators (e.g: SRC-1 and SRC-2 among others), necessary for their transcriptional activity. In this regard, the identification of the dimerization partner of AhR (named Aryl hydrocarbon receptor nuclear transcolator, ARNT) as a coactivator of the ERs (Brunnberg et al. 2003) suggest a novel mechanism by which antiestrogenic effects may occur (Ruegg et al. 2008) (Fig.11A).
- Activated AhR may inhibit E2-induced transactivation through direct binding to specific DNA sequences located near ERE, the inhibitory xenobiotic response elements (i)XREs (Fig. 11B)

- Induction of CYPs may increase E2 metabolism, therefore reducing the estrogenic response. (Fig. 11C).
- AhR ligands may interfere with hormonal signaling by targeting the ERs to the proteasome and thereby enhancing their degradation (Fig. 11 D).

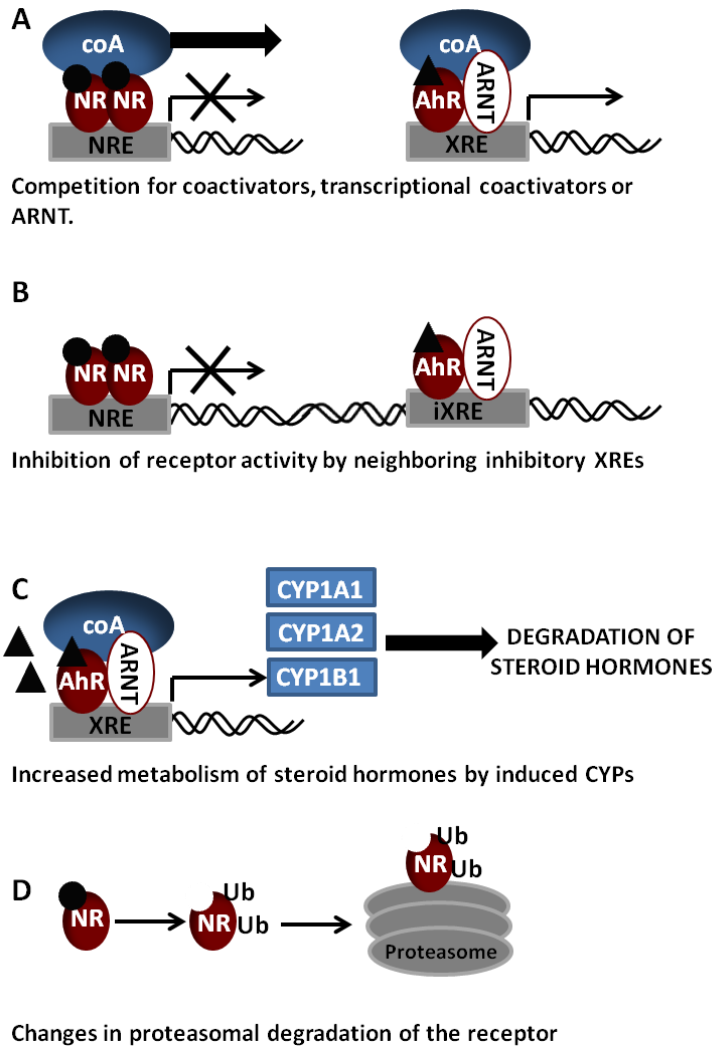


Fig. 11 Possible mechanisms of interference caused by AhR on ER activity.

4. THE IMMUNE SYSTEM: AN OVERVIEW

The immune system refers to the group of biological structures and processes whose ultimate function is to protect the organism against disease. To do so, and by means of complex mechanisms, it identifies and eliminates pathogens (e.g.: virus, bacteria) but also represses the growth of tumors (Magnadottir 2010). In vertebrates, the immune system consists of primary and secondary lymphoid organs. The function of primary organs is to generate lymphoid cells from immature progenitor cells while secondary organs are responsible for lymphocyte maturation. In higher vertebrates, thymus and bone marrow are the primary lymphoid organs, responsible for T and B cells lymphopoiesis, respectively, and spleen, lymph nodes and mucosal associated lymphoid tissue (MALT) constitute the secondary organs. Teleost fish on the contrary, lack lymph node and bone marrow and the lympho-hematopoietic function is assumed by the head kidney.

Traditionally, immune responses have been divided into innate and adaptive mechanisms. The former is an ancient system thought to have appeared hundreds of millions or even billions of years ago (Beutler 2004) and therefore present in all vertebrates and invertebrates. On the contrary, adaptive immunity appeared only about 400-500 millions of years ago (Magnadottir 2010) and it is exclusive of vertebrates. In order to provide an overview here we will briefly described main players in fish immunity.

4.1. Innate immunity of fish

Although it has sometimes been considered more primitive when compared to the adaptive, for most organisms, innate response is enough to defend themselves against disease (Beutler 2004). In the particular case of fish, which are in intimate contact with an environment potentially containing high amount of pathogens, innate immunity is of supreme importance (Ellis 2001). Innate immunity has evolved over the time and includes epithelial/mucosal barrier, and humoral and cellular components, which are below briefly described.

4.1.1.Epithelial and mucosal barrier

Because of the aforementioned reasons, this is an extremely important disease barrier in fish and constitutes the first line of defense (Ellis 2001, Beutler 2004). In addition to provide physical and mechanical protection, piscine mucus contains several immune defenses, including antimicrobial peptides (e.g.: pleurocidin, Syvitski et al. 2005), trypsin-like proteases (e.g.:

cathepsin L and B; Aranishi and Mano 2000), lectins and lysozymes (Ellis 2001, Beutler 2004). For instance, lectins are Ca^{2+}-dependent proteins able to bind to carbohydrates on the surface of bacteria, and thought to act by preventing the attachment. Lysozymes on the other hand are bacteriolytic proteins which attack the bacterial peptidoglycan (Ellis 2001).

4.1.2. Humoral elements of innate immunity.

They are extracellular proteins or molecules able to detect and kill microbes. Among them, the most important defense factor is probably the complement system. It can be activated through all three pathways: the classical (CCP), alternative (ACP) and the lectin (LCP) complement pathways (Rev. in Boshra et al. 2006, Gasque 2004). The CCP is activated by antibody binding to a cell surface although it can also be activated by acute-phase proteins or directly by certain viruses and bacteria. On the contrary, ACP is independent of antibodies and is directly initiated by foreign microorganisms. For instance, it may be activated by lipopolysaccharide (LPS), the major component of cell wall of Gram-negative bacteria, leading to the lysis of the cell wall. ACP activity is very high in fish serum compared to mammals (Lange et al. 2001), indicating the importance of this pathway in fish immunity (Ellis 2001). Thirdly, LCP is activated by the binding of a complex comprised of mannose-binding lectin (MBL) and serine proteases to mannans in bacterial cell surface (Holland and Lambris 2002). These three pathways can convey in membrane attack complex and cell lysis or enhanced phagocytosis by opsonization of the pathogen and activation of the adaptive immune response (Magnadottir 2010).

Other important constituents of the humoral innate immunity include for instance pentraxins. These are pentameric proteins, homologue of mammalian C-reactive protein and serum amyloid P component (SAP), which are central in acute-phase response. In fish serum, pentraxins are present in a high concentration, suggesting that they play a key role in defense mechanisms (Ellis 2001). In addition, some of the proteins occurring in the mucus and previously mentioned are also present in the serum, although at higher concentrations (e.g.: Lysozymes and lectins). For instance, lectins, such as the mannose-binding lectin (MBL) may act as opsonins (Russell and Lumsden 2005) and transferrins prevent bacterial growth by reducing the availability of iron (Ellis 2001, Uribe et al. 2011). Moreover, type I interferon (IFN), which include IFNα and β, is a family of proteins which confer resistance against viral infections (Ellis 2001, Rubio-Godoy 2010). They act by inducing the expression of a number of proteins which inhibit the translation of viral mRNA (Ellis 2001). These include for instance MX proteins, which in

rainbow trout were characterized 20 years ago (Trobridge et al. 1997, Trobridge and Leong 1995). The general term for all these parameters is “pattern recognition proteins or receptors” (PRP/R). They act by recognizing pathogen associated molecular patterns (PAMPs) such as polysaccharides and glycoproteins including LPS, flagellins, peptidoglycan, etc. Important players in PAMP recognition are the Toll-like receptors (TLRs), a family of transmembrane proteins exhibiting a remarkable specificity of the innate immune response (Rev. in Magnadottir 2010). TLRs have been identified in a number of piscine species including zebrafish (Jault et al. 2004) and rainbow trout (Palti et al. 2010) among others (Palti 2011), showing a similar organization and expression than those found in mammals (Bricknell and Dalmo 2005).

4.1.3. Cellular elements of innate immunity

The cellular component of innate response in fish primarily includes highly mobile phagocytic cells (macrophages and granulocytes) and non-specific cytotoxic cells (NCC) (Secombes 1996).

- Macrophages

Literally “large eaters” (Metchnikoff 1893), macrophages are phagocytic cells found in every tissue throughout the organism, where they fulfill two main functions: they kill and engulf microbes, but in addition, they initiate the process of inflammation by secreting a type of signaling molecules named cytokines (e.g.: IL-1; TNF-α…) (see section 4.2.3) that serve to recruit other myeloid cells to the site of infection. In addition, macrophages also act as antigen presenting cells (APCs) to T-cells, involved in adaptive immune response (see 4.2.2 section), (Magnadottir 2010)

- Granulocytes

They include neutrophils, eosinophils and basophils. The most common are neutrophils and eosinophils, while basophils are absent in most piscine species. Similarly to macrophages, neutrophils (and in some cases eosinophils) are phagocytic cells, able to engulf a wide variety of inert and antigenic particles (Secombes 1996).

- Non-specific cytotoxic cells (NCC)

They are considered the functional equivalent of the mammalian natural killer cells (NK). They are able to lyse a variety of transformed mammalian cell lines and seem to play a role in the immune defense against protozoan parasites (Plouffe et al. 2005).

4.2. Adaptive immunity

In contrast to the innate immune system, present in virtually all the organisms, adaptive immunity is restricted to vertebrates, with teleost fish being the oldest living organism presenting this type of immune response (Sunyer 2012). This system can be defined by several key elements: B and T lymphocytes, antigen receptors (immunoglobulins, Ig, and T-cell receptor, TCR), major histocompatibility complex (MHC), genes involved in rearrangement (RAG), somatic hypermutation, gene conversion and primary and secondary lymphoid tissues (Flajnik and Du Pasquier 2004). Bony fish present all these key elements with the exception of the germinal centers (sites where mature B lymphocytes proliferate, differentiate, mutate their antibodies and switch the class of their antibodies) and the Ig class switch (process by which B-lymphocytes switch the expression of Ig class from IgM to IgG, IgE,or IgA).(Flajnik and Du Pasquier 2004). Similarly to the innate response, the adaptive arm is also comprised by both humoral (immunoglobulins) and cellular components (B and T lymphocytes).

4.2.1. Humoral elements of adaptive immunity.

The key humoral component of the adaptive response are immunoglobulins (Ig antibodies), which can be expressed as membrane molecules of B-lymphocytes or secreted into the plasma. In mammals, 5 main classes of immunoglobulins exist (Alberts et al. 2002). Until recently it was generally thought that a tetrameric IgM was the only functional class in teleosts (Salinas et al. 2011). Recent advances in the field of piscine immunology have demonstrated the existence of other isotypes. More specifically, igD (Edholm et al. 2010), and the igT/igZ isotype, present in rainbow trout and zebrafish, respectively (Hansen et al. 2005, Danilova et al. 2005), have been described. Tetrameric igM appears to be the most abundant isotype in fish serum (Sunyer 2012).

4.2.2. Cellular elements of adaptive immunity

Cell-types involved in adaptive immune response are B and T lymphocytes.

- <u>B- lymphocytes</u>

They are involved in the humoral response, secreting antibodies (i.e. Ig), always in presence of macrophages, which secrete IL-1, necessary for this type of response. Teleost fish contain a variety of B-cells subsets which varies according to the species. For instance catfish are known to have three subsets (IgM^+/igD^- ; igM^+/igD^+; igM^-/igD^+) while in rainbow trout only two subsets

have been identified so far ($IgM^+/IgD^+/IgT^-$ and $IgM^-/IgD^-/IgT^+$) (Sunyer 2012). A particular feature of teleost B-cells is their ability to kill and engulf microbes (Sunyer 2013).

In mammals, immature B-cells are produced in the bone marrow but in teleost fish the lymphopoietic and hematopoietic functions take place in the head kidney. Mature naïve B-cells migrate via peripheral blood to other immune tissues such as the spleen. Following antigen (Ag) encounter, they proliferate and differentiate into plasmablasts, which secrete antibodies and initiate the humoral immune response. After several rounds of proliferation, plasmablasts may return to the head kidney, where they differentiate into plasma cells (Bromage et al. 2004).

- T-lymphocytes

In all vertebrates including fish, T-cell development occurs in the thymus. They recognize antigens presented by antigen-presenting cells such as macrophages and are responsible for cell-mediated immunity (Kum and Sekkin 2011). In addition, they are involved in cytokine secretion and also act as helpers of B-lymphocytes (Magnadottir 2010). T-cells recognize antigens only when presented by an antigen presenting cell (APC), which in fish are usually the macrophages, although B lymphocytes may also play a role at this level. All T lymphocytes express T-cell receptors (TCR) and, according to their surface markers, can be further divided into two populations. On the one hand, T cytotoxic (Tc) cells express CD8 surface proteins while T helper (Th) present CD4 markers. $CD8^+$ T cells recognize antigens that are displayed as peptides associated to MHC class I complexes on the surface of all nucleated cells, while $CD4^+$ helper T cells recognize peptides associated with MHC class II only in APCs such as macrophages and B cells. Tc cells directly kill infected cells while Th cells activate B cells to secrete antibodies and macrophages to destroy ingested microbes, but moreover help to activate Tc cells to kill infected target cells.

4.2.3. Cytokines

Cytokines are small polypeptides or glycoproteins (less than 30kDa) secreted, mainly by macrophages and T lymphocytes, in response to an activation of the immune system. They play a key role in cell signaling, with their action being either autocrine (acting on the cells that produce them) or paracrine (acting on nearby cells).

Despite being a heterogeneous group of proteins, which include interleukins, chemokines, interferons, growth factors etc (Dinarello 2007) from the functional point of view they are considered a family. It seems that piscine cytokines possess a similar repertoire to that found in

mammals (Plouffe et al. 2005). Important cytokines described in fish include interleukin-1β (IL-1β), tumor necrosis factor α (TNF-α), interleukin-6 (IL-6) and interferons.

4.3. Immune-neuroendocrine interactions

Increasing evidence accumulated over the last years has suggested that mammalian immune and neuroendocrine systems are closely connected and that a bi-directional relationship between both systems exists and is necessary for the maintenance of homeostasis (Harris and Bird 2000).

A potential immunomodulatory role of hormones in fish has been particularly studied in the case of cortisol. This hormone has depressive effects on a number of immune parameters including phagocytosis, antibody production and lymphocyte proliferation and induces apoptosis in B cells (Rev. in Harris and Bird 2000). Here we will briefly describe main findings and evidences related to the role of sexual and thyroid hormones as well as aryl hydrocarbon receptor (AhR) in fish immunity.

4.3.1. Estrogens, EDCs and immune system.

In mammals, considerable evidence supports that sex hormones are involved in both the innate and adaptive immune response (Nadkarni and McArthur 2013). For instance, estrogens appear to influence neutrophil activation, diminish the release of proinflammatory cytokines from neutrophils and macrophages (Rev. in Nadkarni and McArthur 2013) and inhibit T and B lymphopoiesis (Islander et al. 2003) among other effects (Rev. in Milla et al. 2011, Nadkarni and McArthur 2013). Moreover, the expression of estrogen receptors, ERα and ERβ, on immune organs and T and B lymphocytes (Shim et al. 2006, Pierdominici et al. 2010) suggests that E2 can regulate their responses (Nadkarni and McArthur 2013).

In fish, increasing evidence also suggests an immunomodulatory role of estrogens (Reviewed in Milla et al. 2011). For instance, a number of immune-related genes are expressed in gonadal tissues (e.g.: Chaves-Pozo et al. 2008, Kumari et al. 2009). Conversely, estrogen and androgen receptors are expressed in immune organs and isolated leukocytes (Casanova-Nakayama et al. 2011, Liu et al. 2009), suggesting that these might be targets for sex-steroids. Moreover, estrogen exposure is able to modulate immune gene expression as evidenced by microarray analysis (Casanova-Nakayama et al. 2011, Williams et al. 2007).

Considering all these facts together, the question arises whether estrogen-active EDCs may also target the immune system of fish (Casanova-Nakayama et al. 2011, Milla et al. 2011). In fact, this possibility has been the subject of study of many reports in the last years and accumulating evidence supports the hypothesis. For instance, the well-known EDCs BPA and EE2 modulated the expression of a relatively high amount of immune-related genes in common carp (*Cyprinus carpio*), and provoked a concentration-dependent inhibition of several genes of the complement pathway (Moens et al. 2007). Contradictory data exists on the effect of estrogens and estrogen-like substances on lymphocyte proliferation, with some studies reporting a stimulatory effect (Thilagam et al. 2009, Yin et al. 2007) and others describing the opposite outcome (Cuesta et al. 2007, Schwaiger et al. 2000). Other immune parameters such as antibody production also appear to be influenced by EDCs, with the effects being sometimes contradictory (Rev. in Milla et al. 2011). For instance, in channel catfish (*Ictalurus punctatus*), PCB 126 administration (an ER antagonist) at 0.01 mg/kg caused an increase in the number of specific antibody secreting cells against *Edwardsiella ictaluri* (Rice and Schlenk 1995) but when administered at 1.02 mg/Kg provoked a decrease in plasma antibodies against *Vibrio anguillarum* (Regala et al. 2001). Although these controversial results may be explained by the different species, doses and duration of exposures, in any case suggest that several immune parameters are affected by estrogen exposure. After demonstrating such effects, the subsequent question would be whether these changes in leukocytes, immune-related proteins and antibody production among others, would affect the ability of the animal to fight pathogens (Milla et al. 2011) and therefore compromise their survival. This question has been addressed by (Wenger et al. 2011), which found that the increased mortality of E2-exposed juvenile rainbow trout after infection with *Yersinia ruckeri* co-occurred with a reduced ability to up-regulate several complement genes in response to bacterial infection. Similarly, juvenile rainbow trout exposed to high doses of the EDCs atrazine and nonylphenol exhibited higher mortality following challenge with *Listonella anguillarum* (Shelley et al. 2012).

In contrast, fewer studies have assessed the effect of androgens onto piscine immune system but it seems that they exert anti-proliferative effects on lymphocytes and reduce igM secretion. Moreover, 11-KT has been shown to suppress respiratory burst activity and phagocytosis in kidney macrophages (Rev. in Milla et al. 2011).

4.3.2.Thyroid hormones and immune system.

Similarly to what happens to estrogens, in mammals, the existence of a bi-directional and complex relationship between the hypothalamus-pituitary-thyroid (HPT) axis and the immune system is well accepted (Rev. in De Vito et al. 2011, Klein 2006). For instance, mammalian white blood cells express thyroid receptors (Hastings et al. 1997, Barish et al. 2005) and have been found to contain T3 (Csaba et al. 2004, Pallinger et al. 2005). Moreover, thyroid hormones (THs) influence the distribution of lymphocyte subsets, differentiation of B lymphocytes, proliferation of T lymphocytes, cell-mediated immunity, macrophage maturation, etc (De Vito et al. 2011, De Vito et al. 2012, Perrotta et al. 2014). In addition, immune functions such as phagocytosis, generation of reactive oxygen species (ROS), and synthesis and release of cytokines are affected by hypo- and hyper-thyroid conditions (De Vito et al. 2011). The effect of thyroid status immunity has also been studied in birds. For instance, mallards (*Anas platyrhynchos*) dosed with 125 µg T4 kg^{-1} day $^{-1}$ for 22 days, exhibited reduced antibody production and lectin-dependent cellular cytotoxic activity against tumor cells (Fowles et al. 1997). In contrast, a possible immunomodulatory role of THs has been scarcely explored in fish (Yada and Nakanishi 2002, Escarné et al. 2008) and only few studies have provided indirect evidence that THs influence piscine immune parameters. For instance, hypothyroid fish exhibited significantly reduced numbers of circulating leukocytes (Slicher 1961) while administration of T4 or TSH recovered the number of circulating lymphocytes in hypo-physectomized killifish (Ball and Hawkins 1976). Moreover, T3-fed rohu (*Labeo rohita*) exhibited increased survival to *Aeromonas hydrophil*, suggesting an immunoestimulating role of T3 (Sahoo 2003). More recently, it has been suggested that THs play a role in thymus development. In that study, administration of T4 provoked an increase in thymus size in developing zebrafish while exposure to the anti-thyroid drug methimazole provoked the opposite effect (Lam et al. 2005). Considering that thyroid hormones exert most of their actions after ligand binding to their corresponding receptors (THRs), the finding of high expression of such receptors in immune organs and isolated immune cells might suggest that thyroid signaling occurs in the piscine immune system (Quesada-Garcia et al. 2014).

It is well known that a number of chemicals are able to impact the thyroid homeostasis in fish (see section 3.4). But moreover, some of them are also able to impact the immune system. For instance, Aroclor 1248 (a mixture of PCBs) administration to brown bullhead (*Ameiurus nebulosus*) led to a decrease in bactericidal activity and circulating Ab against *E. ictaluri* followed by higher mortality in a subsequent challenge. Aroclor treated-animals also exhibited decreased

cortisol and T3 (but no T4) plasma levels. However, correlations between hormones and immune-related endpoints could not been found (Iwanowicz et al. 2009).

4.4. Aryl hydrocarbon receptor and immune system.

In animals, interactions between detoxification and immune systems have been widely discussed (Rev. in e.g.: Morgan 2001, Stockinger et al. 2014). Both of them are involved in the ability of organisms to resist to a wide variety of environmental threats such as microorganisms and xenobiotics and many interactions among them have been described (Reynaud et al. 2008). Increasing evidence suggests that AhR has a multitasking role in the immune system (Rev. in Stockinger et al. 2014) and that exposure to AhR ligands may contribute to the development of immune disorders (Veldhoen et al. 2008, Julliard et al. 2014). AhR is highly expressed in cells of the immune system such as myeloid or B cells while in T cells the level of expression varies widely among subsets (Rev. in Stockinger et al. 2014). In addition, the presence of potential xenobiotic responsive elements (XREs) in the upstream sequences of many inflammatory-related genes would suggest an AhR-regulation. Moreover, AhR exhibits physical interaction and mutual repression with the cytokine-activated transcription factor NF-kB (Tian et al. 1999) and its role in T cell differentiation is well established (Veldhoen et al. 2008, Quintana et al. 2008).

With regards to fish, interactions between both systems have been studied at several levels (Reynaud et al. 2008). On the one hand, infection and inflammation leads to a decrease in CYPs expression and associated activities (Rev. in Reynaud et al. 2008). For instance, juvenile carp infected with *Listeria monocytogenes 4b* exhibited decreased detoxification enzyme activities (Chambras 1999). LPS, commonly used to simulate bacterial infection, has also demonstrated its ability to modulate detoxification activities, although in an organ-specific manner (Rev. in Reynaud et al. 2008). In second place, some AhR ligands are able to provoke deleterious effects on piscine immune system. For instance, a single benzo-*a*-pyrene (BAP) injection depressed lymphocyte proliferation in Japanese medaka (*Oryzias latipes)* (Carlson et al. 2002) while TCDD suppressed antibody production against sheep red blood cells in Chinook salmon (Arkoosh et al. 1994). Lastly, CYP system may transform some non-immunotoxic xenobiotics into highly immunotoxic metabolites.

II. OBJECTIVES

Objectives

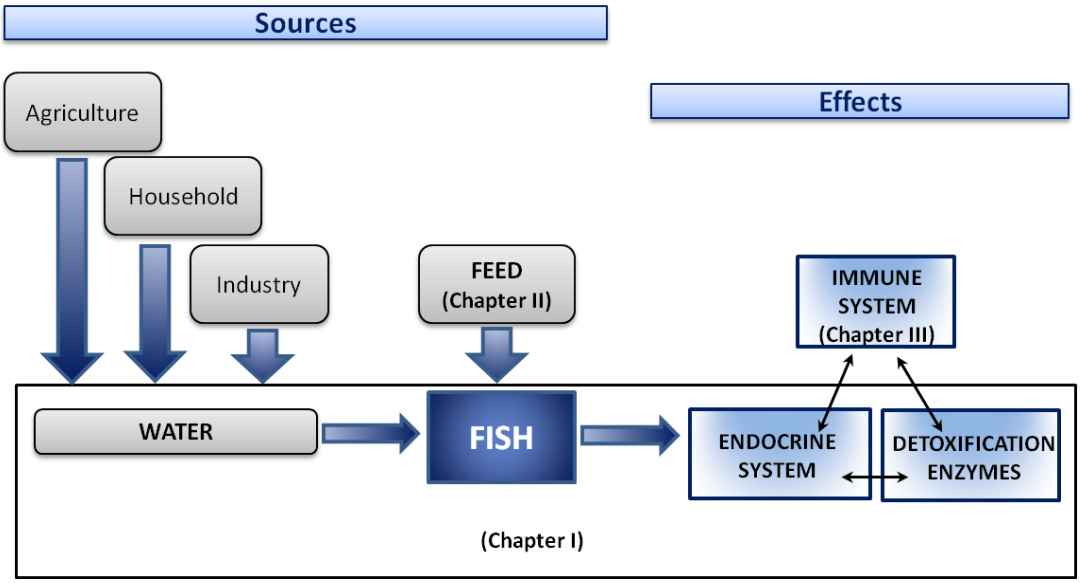
The general objective of this doctoral thesis was to evaluate, from an integrated perspective, the effects of low concentrations of pollutants and endocrine disrupting compounds (EDCs) on farmed fish. This will allow the use of a similar approach in the assessment of potential effects of pollutants on wild fish and on ecosystems.

Taking into account the two main sources of pollutants (water and feed), this general objective has been achieved through the following subobjectives, addressed along the different chapters:

- To identify and evaluate the effect of EDCs and micropollutants present in the waters feeding two fishfarms by using an integrated approach which combines *in vivo* biomarkers (EROD and BFCOD activities), active biomonitoring, and analytical techniques (GC x GC-TOF-MS) (**Chapter I**).

- To assess the presence of EDs (estrogenic and thyromimetic) in commercially available fishfeeds using two *in vitro* reporter assays (**Chapter II**).

- To evaluate whether the exposure to thyroid axis disruptors has detrimental effects on the immune system of fish (**Chapter III**).



Schematic overview of the thesis' objectives, addressed along the different chapters

III. RESULTS

CHAPTER I:
Environmental monitoring of low levels of pollutants by a combination of biological and analytical techniques.

PAPER I:

DETECTION OF EFFECTS CAUSED BY VERY LOW LEVELS OF CONTAMINANTS IN RIVERINE SEDIMENTS THROUGH A COMBINATION OF CHEMICAL ANALYSIS, IN VITRO BIOASSAYS AND FARMED FISH AS SENTINEL

RESUMEN

Los organismos acuáticos se encuentran a menudo expuestos a mezclas de contaminantes en baja concentración cuya presencia y efectos pueden pasar inadvertidos si solo se emplean estrategia de monitoreo tradicionales. El objetivo principal de este trabajo era evaluar la presencia y efecto de niveles traza de contaminantes en un área con un bajo impacto antropogénico por medio de la combinación de aproximaciones químicas y biológicas. Se recogieron sedimentos a lo largo de un río con una escasa presión antropogénica y se midió la actividad EROD con la línea celular RTG-2 procedente de gónada de trucha. En esos mismos sedimentos, se realizaron análisis químicos utilizando un cromatógrafo de gases bidimensional acoplado a espectómetro de masas por tiempo de vuelo (GCx GC-TOF-MS). Las muestras indujeron actividad EROD y los análisis químicos evidenciaron la presencia de una gran cantidad de contaminantes en concentraciones de ng/g. La correlación entre la inducción EROD y los datos del análisis químico mostraron una r de 0.840 ($p<0.05$). A ello se suma que los peces procedentes de una piscifactoría localizada aguas abajo de los puntos de muestreo mostraron niveles de EROD hepáticos elevados así como una sobreexpresión de los citocromos cyp1a y cyp3a. En conclusión, solo la apropiada combinación de técnicas biológicas y químicas permitieron detectar la presencia de niveles traza de contaminantes en un río teóricamente sin impacto/no contaminado.

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Detection of Effects Caused by Very Low Levels of Contaminants in Riverine Sediments Through a Combination of Chemical Analysis, In Vitro Bioassays, and Farmed Fish as Sentinel

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Abstract Aquatic organisms are often exposed to mixtures of low levels of pollutants whose presence and effects can pass easily unnoticed if only traditional monitoring strategies are employed. The main aim of this work was to assess the presence and effects of trace levels of pollutants in a scarcely affected area through the combination of chemical and biological approaches. Sediments were collected along a river with little anthropogenic pressure and assayed for cytochrome P450 (Cyp1a)-dependent ethoxyresorufin-*O*-deethylase (EROD) activity with the rainbow trout gonadal cell line RTG-2. Chemical analyses were performed in these sediments using two-dimensional gas chromatography-time-of-flight mass spectrometry. Sediment samples induced EROD activity, and chemical analyses evidenced the presence of a wide variety of contaminants in the range of nanograms per gram of dry weight. Correlation analysis between EROD induction and chemical analyses data showed an r value of 0.840 ($p < 0.05$). In addition, fish from a fish farm located downstream of the sampling points exhibited high hepatic EROD levels as well as an induced expression of *cyp1a* and *cyp3a*. In conclusion, only an appropriate combination of biological and chemical techniques allowed the detection of the presence of trace levels of contaminants in a theoretically nonaffected river.

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During the last century, water quality has suffered a strong deterioration, mainly due to chemical contamination caused by human activity. The tremendous progress experienced by analytical sciences in the last years has allowed the detection and quantification of low or even trace concentrations (ng/l range) of a wide variety of substances in almost any water body studied (Petrovic et al. 2005; Alonso et al. 2010). Although chemical analyses are essential for monitoring purposes, they do not allow the prediction of global effects of mixtures on biota because they are not able to detect additive, antagonistic, or agonistic activities undoubtedly present in the pool of analyzed substances. Similarly, there can be a very high number of substances present in the environment at levels lower than detection limits that probably are not responsible of deleterious effects individually but that exert effects when considered together.

In this context, a cost-effective method to monitor these effects of chemicals on biota is based on the use of

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biomarkers. Those are defined as physiological or histological changes that are indicative of exposure to or effects of xenobiotics at organismal and suborganismal levels (Mayer et al. 1992). Among them, the induction of mixed-function oxidases, such as cytochrome (Cyp) P450, is frequently used.

CYP P4501A (Cyp1a) plays a key role in the biotransformation of many xenobiotics. It is activated in presence of a broad variety of chemicals; as a consequence, it has been widely used as an in vivo biomarker of environmental exposure (Nebert et al. 2000) measured either at the protein (Sturve et al. 2006), gene expression (Kim et al. 2013), or enzymatic level. One of the enzyme activities dependent on Cyp1a and commonly used for monitoring purposes is ethoxyresorufin-*O*-deethylase (EROD) (Jung et al. 2014). Cyp1a induction is mediated by way of the aryl hydrocarbon receptor (Ahr) (Stegeman et al. 1995), which is a ligand-dependent transcription factor that regulates the expression of a battery of genes (Nebert et al. 2000). Typical Ahr agonists are planar, polycyclic compounds including polyaromatic hydrocarbons (PAHs), polychlorinated dibenzodioxins, and polychlorinated biphenyls (PCBs) (Denison and Nagy 2003). However, an increasing number of data indicate that Ahr can also be activated by other structurally different compounds including some drugs (Fernandez-Cruz et al. 2011), pesticides (Casado et al. 2006), and even natural occurring substances (Behrens and Segner 2005). For instance, some compounds associated with wood products and humic substances present in pulp mill effluents, such as retene or juvabione, have been found to induce EROD activity (Fragoso et al. 1998; Martel et al. 1997; Matsuo et al. 2006).

In addition, the presence of Ahr agonists in a given environmental sample can also be detected in vitro on primary cultures or on permanent cell lines expressing a functional form of Cyp and Ahr. This approach has shown its usefulness in ecotoxicological hazard assessments of a number of complex environmental samples including sediments (Wang et al. 2014a; Qiao et al. 2006), wastewater effluents (Smital et al. 2011), and drinking water samples (Wang et al. 2014b).

In addition to its role on detoxification pathways, Ahr has also been linked to endocrine-disrupting effects. More specifically, the activation of Ahr is able to modulate estrogen receptor (Er) signaling pathway (Swedenborg and Pongratz 2010) leading to (anti)estrogenic effects (Navas and Segner 1998). For instance, in vitro exposure of fish hepatocytes to PAHs caused a decrease in the production of 17-ß-estradiol (E2)-induced vitellogenin (Vtg) (Navas and Segner 2000), an oestrogen-dependent lipoglycophosphoprotein, which serves as the precursor to egg yolk (Mommsen and Walsh 1988) in oviparous vertebrates. This effect has also been observed in vivo (e.g., Anderson et al. 1996; Palumbo et al. 2009; Bemanian et al. 2004) and in field studies (Valdehita et al. 2012).

Another Cyp that in recent years has shown its usefulness as a biomarker in environmental monitoring studies is Cyp3a (Della Torre et al. 2010; Quesada-Garcia et al. 2013). This cytochrome is also implicated in detoxification processes and due to its broad substrate specificity is probably one of the most important drug-metabolizing enzymes in vertebrates contributing to the metabolism of approximately 50 % of currently marketed drugs (Guengerich 1999). In mammals, its expression is regulated by the pregnane-X-receptor (Pxr), a highly promiscuous nuclear receptor that allows the efficient elimination of structurally different chemicals (Lehmann et al. 1998). However, much less is known about piscine Pxr, which seems to be regulated in a different manner than in mammals and has proved highly unresponsive to prototypical mammalian ligands (Wassmur et al. 2010).

Traditionally, environmental biomonitoring using fish has involved either the capture of individuals (Della Torre et al. 2010; Jebali et al. 2013) or their caging (Browne et al. 2010; Rodriguez-Fuentes et al. 2012). However, the first approach has several disadvantages, including the ability of fish to migrate for feeding or breeding purposes to avoid pollution (Svecevicius 1999) or the generation of resistance mechanisms as a result of selective pressures (Wirgin et al. 2011). Regarding the second strategy, maintenance of organisms in reduced spaces can be an alternative source of stress that must be considered (Oikari 2006) and that may alter the results of the study. As an alternative, the use of fish farms to assess water quality has recently emerged and proved its usefulness (Quesada-Garcia et al. 2013; Valdehita et al. 2012).

The present study aimed (1) to assess the presence of Ahr inducers in sediment samples collected along a lowly affected river using a combination of biological and chemical analyses (EROD bioassay and two-dimensional gas chromatography-time-of-flight mass spectrometry (GC×GC–TOF–MS) and (2) to evaluate the possible effects of the very low levels of contaminants detected on fish [rainbow trout (*Oncorhynchus mykiss*)] located in a fish farm fed by the river. By showing these subtle effects in farmed fish (effects that do not alter the final quality of the product and that do not affect the economical suitability of the exploitation), we also wanted to explore the usefulness of fish farms as a possible tool for monitoring and guaranteeing the quality of river waters.

Materials and Methods

Chemicals

All chemicals were purchased from Sigma–Aldrich (California, USA) unless otherwise noted. Tris-HCl, sucrose, and KCl were purchased from Panreac Quimica S.L.U.

(Zaragoza, Spain). The iScript RT-PCR kit with SYBR Green was from Bio-Rad (California, USA). L-glutamine (200 mM), fetal bovine serum (FBS), penicillin and streptomycin (10,000 U ml^{-1}), no essential amino acids 100× (NEAA), and EMEM (Eagle's minimum essential medium) cell culture medium were from Lonza (Barcelona, Spain). Analytical-grade methanol and sodium chloride (purity 99.5 %) were supplied by J. T. Baker (Deventer, Holland). Analytical-grade water was purchased from Fluka (Buchs, Switzerland).

Sediment Samplings Along The River

A sediment sampling campaign was performed during the summer season in a river located in the northern region of Spain (the autonomous community of Castile and León) and its two tributary branches. The watercourses flow through an isolated area with minimal anthropogenic pressure and feed a rainbow trout fish farm located approximately 12 km downstream. Seven sediment samples, belonging to four towns, were taken in points of interest such as before and after towns or wastewater-treatment plants (WWTPs) (see Table 1). Samples were extracted as described later in the text and subjected to cellular assay (see section "EROD Activity and Protein Measurements in RTG-2 Cells") and chemical analyses (see section "Chemical Analyses in Sediments Along the River").

Sediment Extraction

All sediment samples were taken from the top layer of the river bed (0–7 cm), where pollutants are expected to accumulate. The extraction of chemicals from sediments was performed using hexane as described elsewhere (Valdehita et al. 2012). Final extracts consisted of 250 µl of dimethyl sulfoxide (DMSO) with a concentration of 4 g sediment ml^{-1} DMSO. The extracts were maintained at −20 °C until their assessment in the RTG-2 cell line or chemical analyses. To prevent any degradation of the sediment samples, they were stored at −80 °C immediately after arrival at the laboratory. Extracts were obtained and applied to cells between 2 and 4 weeks later.

Cell Culture

The fibroblast-like RTG-2 cell line from *O. mykiss*, was obtained from the American Type Culture Collection. Cells were cultured in EMEM supplemented with 1 % penicillin/streptomycin, 10 % FBS, 1 % L-glutamine, and 1 % NEAA. Cultures were grown at 20 °C in a humidified 95 % air/5 % carbon dioxide atmosphere and split weekly with 0.5 % trypsin/0.02 % ethylene diamine tetraacetic acid).

EROD Activity and Protein Measurements in RTG-2 Cells

The presence of contaminants in the sediment extracts was evaluated by the induction of EROD activity in RTG-2 cells exposed to different extract dilutions. Cells were cultured in 96-well plates (Greiner Bio-One GmbH, Frickenhausen, Germany) at a density of 2.5×10^4 cells/well. After 24 h, cells were exposed to different concentrations of sediment extracts (2.5–40 mg extract/ml medium). β-naphthoflavone (βNF), a prototypical Ahr ligand and EROD inducer, was used as positive control at concentrations ranging from 1.95 nM to 0.25 µM (0.53–68 ng ml^{-1}, respectively).

βNF and sediment extract stock solutions were diluted in culture medium at a maximal solvent (DMSO) concentration of 1 % (v/v), a concentration that has no effect on cell viability or EROD activity (data not shown). Control cells received the maximum DMSO concentrations used in the treated cells. Twenty-four hours after treatment, medium was removed, and cells washed with phosphate-buffered saline (pH 7.5) and lysed with liquid nitrogen. EROD activity was measured according to the method described by Burke and Mayer (1974) and normalized to the protein concentration determined using the fluorescamine assay as

Table 1 Location of sampling sites

Sites	Population (inhabitants) in 2014	Sample ID	Sampling site location	Distance upstream to fish farm (km)
Town 1	775	SS1	Upstream town	8.8
		SS2	Downstream town	6.7
Town 2	877	SS3	Upstream WWTP	11.3
		SS4	Downstream WWTP	9.4
Town 3	2,274	SS5	Upstream WWTP	6
		SS6	Downstream WWTP	4.5
Town 4	0	SS7		4

previously described (Kennedy et al. 1995; Navas and Segner 2000).

In addition, based on the results of the chemical analyses (see section "Chemical Analyses"), RTG-2 cells were exposed to increasing concentrations of 4 different PAHs (pyrene, fluoranthene, anthracene, and naphtalene) and 3 chemicals commonly included in personal care products (PCPs): 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethyl-cyclopenta-[*g*]-2-benzopyrane (HHCB), 6-acetyl-1,1,2,4,4,7-hexamethyltetralin (AHTN), and 5-chloro-2-(2,4-dichlorophenoxy)phenol (triclosan). Cells were exposed to each of the chemicals alone and also in combination simulating three different mixtures (four PAHs, three PCPs, and four PAHs+ three PCPs [mixture]). Maximum testing concentrations were selected based on the chemical analyses results (maximum concentration found in samples multiplied by a factor of 15,000). From that concentration, 18 serial dilutions (1:2) were performed (Table 2).

Chemical Analyses in Sediments Along the River

Samples of sediments were extracted by solid–liquid extraction as previously described (Valdehita et al. 2012) and analyzed by GC×GC–TOF–MS (Gómez et al. 2011). Using this method, nonpolar target compounds (10, 15 PAHs, and 9 pesticides) were quantified (Online Resource 1). The GC×GC–TOF–MS system was an Agilent 7890A (Agilent, Palo Alto, California, USA) gas chromatograph, equipped with a secondary oven to fit the secondary column, and a quad-jets modulator (two cold jets and two hot jets). The first column was an Rtx-5 (10 m × 0.18 mm i.d., 0.2 μm), and the second column was an Rxi-17 (1 m × 0.1 mm i.d., 0.10 μm) (both from Restek, Bellefonte, Pennsylvania, USA). High-purity helium (flow rate 1.5 mL/min) was used as carrier gas. The chromatographic conditions were as follows: the first-dimension column oven temperature program began at 70 °C for 3 min, then increased to 150 °C at a rate of 30 °C/min, followed by a 5 °C/min ramp to 200 °C, and finally at a rate of 15 °C/min to 285 °C, and held at this temperature for 5 minutes. The total analysis time was 27.17 minutes. The second-dimension column oven temperature began at 15 °C, then 20 °C, and finally 30 °C greater than the corresponding first-dimension column oven temperature with the same rate and hold time. The MS system was a Pegasus 4D TOF from LECO Corporation (St. Joseph, Michigan, USA). Electron impact mass spectra in full-scan mode were obtained at 70 eV. The temperature of the transfer line and the ion source were set at 280 and 250 °C, respectively. The TOF–MS was run in the range of 50–450 *m*/*z* at an acquisition rate of 100 spectra/s with a detector voltage of 1,460 V. Auto-tuning for TOF–MS optimization was performed before each optimization test sequence.

Instrument control and data processing were performed with the Leco ChromaTOF (version 4.24) software. Data processing included automatic peak-finding using MS deconvolution. Quantification was performed through matrix-matched calibration curves. In addition, spectral searches against the National Institute of Standards and Technology 2008 and Wiley mass spectral libraries were performed.

The total 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalents (TEQs) for PAHs were calculated from the chemical analysis results by multiplying the concentration of each PAH in the mixture by its corresponding individual toxic equivalency factor (TEF) and then summed as suggested by the World Health Organization (Van den Berg et al. 2006). For the PAH-TEFs, the values of Collins et al. (1998) for human risk assessment were used. The results were expressed as pg TEQ g^{-1} of sediment.

Fish Samplings in the Fish Farm

A rainbow trout farm receiving waters from this river (farm A) was sampled in September, October, November, and December. Parallel samplings were performed in another fish farm used as reference (farm B), which is located in Castile-La Mancha, far away from any anthropogenic pressure and receives theoretically very clean waters. In each sampling, water temperature, pH, and oxygen saturation were recorded. Rainbow trout captured in farm B were the same sex and age as those from farm A. In each farm, eight female *O. mykiss* were anaesthetized by tricaine methane sulphonate and killed. Fork length (LF) and mass

Table 2 Substances and range of concentrations tested in the EROD assay using the RTG-2 cell line

Substance	Minimum concentration tested (μM)	Maximum concentration tested (μM)
Anthracene	3.05E−06	0.4
Naphthalene	1.11E−05	1.45
Fluoranthene	9.77E−06	1.28
Pyrene	1.02E−05	1.33
HHCB	1.28 E−04	16.78
AHTN	1.63E−05	2.14
Triclosan	2.69E−05	3.52

(M) were recorded, and liver and gonads were weighted. The hepatosomatic and gonadosomatic indexes and the condition factor (HI, GI, and K, respectively) were calculated according to (Gómez et al. 1999) and Rikardsen and Elliot (2000), respectively (Table 3). A sample of liver was immediately processed for RNA isolation. The rest of the liver was immediately frozen in liquid nitrogen and stored at −80 °C until enzyme activity measurements.

EROD Activity in Trout Liver

The measurement of EROD activity in trout liver was performed on the cellular microsomal fraction. Microsomes were isolated as previously described (Valdehita et al. 2012). EROD activity was measured according to the same method as in the cell culture [originally described by Burke and Mayer (1974)] using a 96 well–plate format. EROD activity was normalized to the protein content and measured with fluorescamine (Navas and Segner 2000; Kennedy et al. 1995). EROD was measured three independent times for each individual sample, and each measurement was performed in triplicate.

RNA Extraction from Rainbow Trout Liver and Single-Step Reverse Transcriptase Quantitative Polymerase Chain Reaction

Total RNA was extracted from approximately 0.1 mg *O. mykiss* liver using TRI reagent according to the manufacturer's instructions. RNA concentrations were estimated by Nanodrop and the presence of 18 S and 28 S bands confirmed by denaturing RNA agar electrophoresis (Sigma). Reverse transcriptase–quantitative polymerase chain reaction (RT-qPCR) analysis was performed with 100 ng of RNA for each sample using an iScript RT-PCR kit with SYBR Green in a one-step RT-qPCR protocol according to the manufacturer's instructions. The thermal cycling conditions were 10 min at 50 °C for the reverse transcription, 5 min at 95 °C to inactivate the reverse transcriptase, and 35 cycles of amplification (97 °C for 15 s, 48 °C for 30 s, and 72 °C for 1 min). All PCR reactions were performed in a line-gene K system (Bioer Technology, Hangzhou, China) with specific primers for *cyp1a*, *cyp3a*, *ahr*, *er*, and *vtg* (Table 4). The relative quantification of the target gene was normalized to *β-actin*, which was chosen as housekeeping gene (Valdehita et al. 2012). *β-actin* mRNA expression was constant among the fish cohorts with average values for Ct *β-actin* mRNA of 19.25 ± 0.26. Thus, *β-actin* was considered an appropriate reference gene for normalization in this study. Results of quantitative PCR were represented as Ct values where Ct was defined as the threshold cycle number at which product is first detected by fluorescence. Relative quantification was measured using the comparative Ct method, which is also referred to as the $2^{-\Delta\Delta Ct}$ method, representing the amount of target normalized to the endogenous control (*ß-actin*) and relative to the mean value from an external set of trout (obtained from a second fish farm [farm B]) where $\Delta\Delta Ct = (Ct_{target} - Ct_{ß\text{-}actin})_{fish\ farm\ A} - (Ct_{target} - Ct_{ß\text{-}actin})_{fish\ farm\ B}$. The fold change in relative expression was then determined by calculating $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen 2001). Fish from farm B, used as external cohort, were of the same sex and age and were sampled at the same time that fish in the target farm to avoid confounding factors. All of the experiments were repeated three times.

Statistics and Analysis of Results

For the cell-exposure experiments, results represent the mean of three independent experiments ±SEM with all analyses performed in duplicate within each experiment. The effective concentration causing the 50 % of the maximal response (EC_{50}) was calculated by fitting the EROD assay results to a regression model equation for a sigmoid curve as follows:

$$Y = Y_0 + A[1 + (XX_0^{-1B}]^{-1},$$

Table 3 Mean ± SEM ($n = 8$), M, LF, HI and GI, and K from *O. mykiss* in each sampling

Sampling	Farm	Weight (g)	L_F (cm)	HI	GI	K
September	A	422.93 ± 27.84^{a}	34.00 ± 0.68	0.82 ± 0.02	0.15 ± 0.02^{a}	1.07 ± 0.03
	B	404.08 ± 31.62^{AB}	31.66 ± 0.67	$1.32 \pm 0.09^{AB*}$	1.91 ± 0.61	1.03 ± 0.03
October	A	419.38 ± 21.73^{a}	34.06 ± 0.55	0.86 ± 0.08	0.15 ± 0.01^{a}	1.05 ± 0.02
	B	378.75 ± 16.84^{A}	32.31 ± 0.57	$1.36 \pm 0.10^{A*}$	2.80 ± 0.31^{A}	1.12 ± 0.03
November	A	433.31 ± 37.79^{ab}	35.09 ± 0.79	0.95 ± 0.07	0.28 ± 0.06^{ab}	0.98 ± 0.05
	B	499.56 ± 22.4^{B}	35.30 ± 0.66	$1.31 \pm 0.16^{A*}$	$14.42 \pm 1.76^{B*}$	1.14 ± 0.04
December	A	514.50 ± 43.89^{b}	35.75 ± 1.21	0.87 ± 0.06	0.38 ± 0.04^{b}	1.11 ± 0.03
	B	$377.13 \pm 29.47^{A*}$	31.75 ± 0.61	$0.95 \pm 0.07^{B*}$	$18.74 \pm 1.79^{C*}$	1.17 ± 0.17

Significant differences between farms at a given sampling time are indicated (*$p < 0.05$). Significant differences among samplings for the same fish farm are indicated by lower-case (farm A) or upper-case (farm B) letters ($p < 0.05$)

Table 4 Primers for quantitative PCR analysis of gene expression

Target gene	Primers sequence (5′…3′)	Product size (bp)	Reference
β-actin	Sense: CATCACCATCGGCAACGA	137	Valdehita et al. (2012)
	Antisense: GATGTCCACGTCACACTTCATGA		
Cyp1a	Sense: TCAACTTACCTCTGCTGGAAGC	68	Rees and Li (2004)
	Antisense: GATGAACGGCAGGAAGGA		
Cyp3a	Sense: TCTACCCTGCTGAGCGGAA	198	Quesada-García et al. (2013)
	Antisense: ACAGTGGGTTGAACAGGTCG		
Ahr2	Sense: GTGTTCTATGCCTCTCCTACTATC	90	Valdehita et al. (2012)
	Antisense: GTCATCTGTGTGGATCAGCTCAA		
Er2β	Sense: CTGACCCCAGAACAGCTGATC	125	Nagler et al. (2007)
	Antisense: TCGGCCAGGTTGGTAAGTG		
Vtg	Sense: GCTGCCCTTGATGAGAACGAC	158	Tilton et al. (2006)
	Antisense: TCCCAAGACAACCTCAGACGA		

where A is the maximal response observed, B is the slope of the curve, X_0 is the EC_{50} valu,; and Y_0 is the minimal response. From the biological analysis, ßNF-equivalents, which represent the sediment concentration that provoked the same response as the EC_{50} of the positive control (i.e., ßNF), present in a given sediment sample were calculated. In other words, they were calculated by clearing from the equation of the sigmoidal curve generated by dilutions of the sediment samples the concentration of sediment (X) that induced the same answer (Y) as this of the EC_{50} value of ßNF.

To investigate the association between chemical (expressed as TEQs) and biological analyses (expressed as ßNF-Equivalents), Pearson correlation analysis was performed. Significant differences were accepted at $p < 0.05$.

Concerning the results from EROD activity and RT-qPCR in trout livers, for each sampling time they are expressed as the mean of the results obtained in eight trout ±SEM. The effects of farm and sampling dates were assessed by two-way analysis of variance *followed by post hoc* Tukey's multiple comparison test. All statistical analyses were performed using Sigma Plot 12.0 (San Rafael, California, USA). Sigma Plot 12.0 automatically checks the homogeneity of variances by observing the variability among group means, and Kolmogorov–Smirnov test was used to observe the normality of the distribution. Significant differences were accepted at $p < 0.05$.

Results

Sampling Along the River

EROD Activity Induced by Sediment Extracts in RTG-2 Cells

The presence of substances able to induce EROD activity in the sediments was tested in RTG-2 cells. In each assay, a positive control (βNF) was included to assess the proper functioning of the system. βNF provoked a dose-dependent increase in EROD activity with maximum response (101 ± 7 pmol mg protein^{-1} min^{-1}) reached at a concentration of 6.80×10^{-5} mg ml^{-1}. The calculated EC_{50} value for βNF was 4.87×10^{-6} mg ml^{-1} (Fig. 1).

Regarding the sediment samples, the equivalent concentrations to which cells were exposed ranged from 1.25 to 40 mg sediment ml^{-1}. In most sediments, maximum EROD activity was achieved at an equivalent concentration of 10 mg sediment ml^{-1}. The exceptions were samples SS1, SS3, and SS4, where maximum activity was observed at concentrations ranging from 20 to 40 mg sediment ml^{-1} (Table 5). Measured EROD values ranged between 39 ± 13 (SS1) and 122 ± 14 (SS6) pmol mg protein^{-1} min^{-1} (Fig. 2; Table 5). All but one sediment (SS1) induced a full dose-response curve (Fig. 2), thus allowing the calculation of βNF-equivalents, which ranged between 0.49 (SS7) and 4.09 (SS2) ng/mg sediment (Table 5).

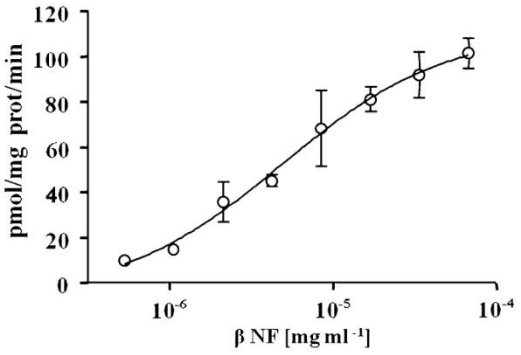

Fig. 1 Dose-response increase of EROD activity in *O. mykiss* RTG-2 cells after a 24-h exposure to βNF (positive control). Each point is the mean ± SEM of five independent experiments performed in duplicate

Chemical Analyses

Chemical analyses in the sediments showed the ubiquitous presence of low concentrations of four PAHs in the study area (anthracene, naphthalene, pyrene, and fluoranthene) at concentrations ranging from 0.06 to 0.45 ng/g (Table 6). The TEQs of each sediment sample were calculated by adding the TEFs for each individual PAH detected in the sample. SS2 exhibited the highest value (2.17 pg TEQ/g dw) and SS1 (0.16 pg TEQ/g dw) the lowest (Table 6).

Regarding the PCPs, HHCB (trade name Galaxolide) was detected in three samples at concentrations ranging from 2.25 to 7.23 ng/g dw, always downstream of the towns, evidencing that it is a good marker of anthropogenic impact. Other PCPs, such as triclosan and AHTN (trade name Tonalide), were also occasionally detected in concentrations in the range of nanograms per gram.

Correlation Between βNF-Equivalents and TEQs

Pearson correlation analysis was performed to investigate how calculated βNF-equivalents (derived from biological assay with RTG-2) and TEQs (calculated from chemical analysis) correlated. When TEQs (y-axis) were represented against ßNF-equivalents (x-axis), statistical analysis showed a significant correlation between both end points ($r = 0.840$, $p < 0.05$) (Fig. 3), thus indicating a positive association. The slope of the adjusted line ($m = 0.281$) evidenced a greater value of the ßNF-equivalents with respect to the TEQ values.

EROD Activity Induced by PAHs and PCPs

None of the tested PAHs or PCPs was able to induce EROD activity in RTG-2 at the tested doses. The combination of substances in complex mixtures (four PAHS, three PCPs, and four PAHs+ three PCPs) did not induce EROD activity in the cells (data not shown).

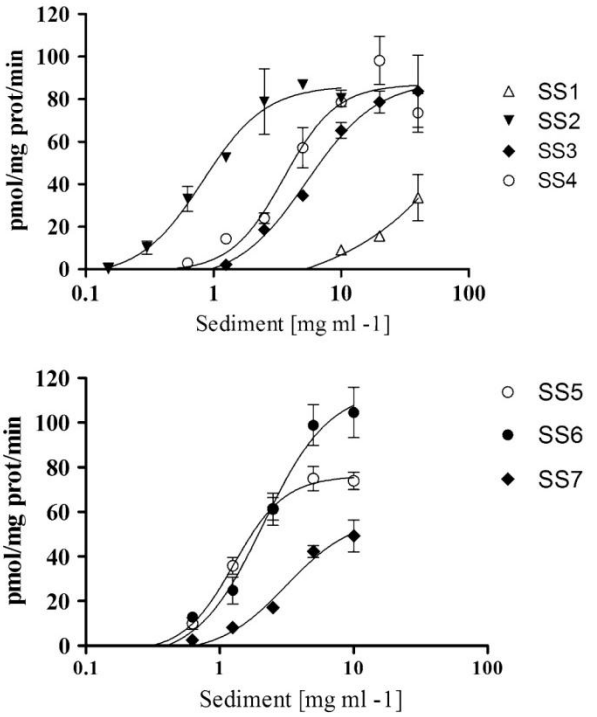

Fig. 2 Dose-response increase of EROD activity in *O. mykiss* RTG-2 cells after a 24-hour exposure to sediment extracts from the river feeding the fish farm A (sampling sites SS1 to SS7). Each point is the mean ± SEM of three independent experiments performed in duplicate

Fish Farm Samplings

Water Conditions, HI and GI, and K

Water pH values were similar in all samplings and ranged from 7.4 to 8.4. Oxygen saturation in the water was always 100 % air saturation. The maximum water temperature was recorded in September (13.0 °C in farm A and 10.1 °C in farm B) and the minimum in December (9.8 °C in farm A and 7.4 °C in farm B) in accordance with the normal

Table 5 Maximum EROD activity, EC_{50} values, and calculated βNF-equivalents in sediment samples along the river feeding the fish farm

Sampling	Cmax (mg sediment/ml)	Maximum EROD (pmol/mg prot min)	EC_{50} (mg sediment/ml)	EC_{20} (mg sediment/ml)	Equivalents ng Eq βNF/mg sediment
SS1	40	39 ± 13	NDR	NDR	–
SS2	10	94 ± 5	0.82	0.38	4.09
SS3	40	98 ± 20	5.42	2.26	0.66
SS4	20	115 ± 13	3.55	1.84	1.11
SS5	10	80 ± 4	1.28	0.70	2.57
SS6	10	122 ± 14	2.12	0.96	2.34
SS7	10	56 ± 9	3.05	1.41	0.49

NDR no dose response

Table 6 Concentrations of substances detected by GC×GC–TOF–MS in sediments (0–7 cm) along the river feeding the fish farm (ng/g dw)

	Town 1		Town 2		Town 3		Town 4
	Ss1 Upstream	Ss2 Downstream	Ss3 Upstream	Ss4 Downstream	Ss5 Upstream	Ss6 Downstream	Ss7
Anthracene	–	0.12	0.06	0.07	0.11	0.1	0.08
Naphthalene	0.16	0.09	0.22	0.16	0.21	0.12	0.31
Fluoranthene	–	0.43	0.23	–	0.26	0.14	–
Pyrene	–	0.45	0.26	–	0.31	0.21	0.22
HHCB	–	7.22	–	2.25	–	–	2.86
AHTN	–	0.92	–	0.21	–	–	–
Triclosan	–	1.7	–	–	–	–	–
TEQs (pg/g dw)	0.16	2.17	1.31	0.86	1.88	1.47	1.33

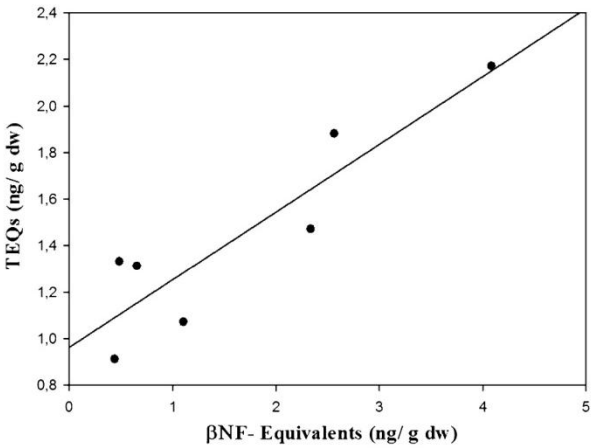

Fig. 3 Fitting the linear regression *curve* of βNF-Eq (ng/g dw) versus TEQs

seasonal variation. In farm A, no differences between samplings were found for fork length (L_F), HI, or *K*. Only weight and GI were significantly greater in December compared with September and October (Table 3). The increase in GI was related with the increase in gonadal size observed in some fish sampled in this month when the reproductive period normally begins. In farm B, there were significant differences among samplings for both HI and GI, with a gradual and significant increase in GI observed along the sampling campaign, due also to the beginning of the reproductive period. When comparing both farms, HI was significantly lower in farm A at all sampling times, whereas GI only showed differences in the last two samplings (November and December).

EROD Activity in Trout Liver

EROD activity was significantly greater in farm A compared with farm B at all sampling times ($p < 0.001$). In farm A, in the first two samplings (September and October) EROD activity values were 365 ± 67 and 387 ± 39 pmol mg protein^{-1} min^{-1}, respectively (Fig. 4). In the following samplings in this farm, EROD activity was significantly lower. More specifically, in November levels were almost 3 times lower than in the preceding sampling (131 ± 16 pmol mg protein^{-1} min^{-1}), whereas in December measured levels were 206 ± 50 pmol mg protein^{-1} min^{-1}. In farm B, no statistical differences among samplings were found. Here, EROD values ranged from 22 ± 12 to 85 ± 18 pmol mg protein^{-1} min^{-1} in November and December, respectively.

Expression of cyp1a and ahr2 in Trout Liver

The *cyp1a* and *ahr2* mRNA levels were assessed by RT-qPCR to investigate whether the increased EROD activity in the livers from *O. mykiss* in farm A was accompanied by an enhancement of the expression of these two genes. For

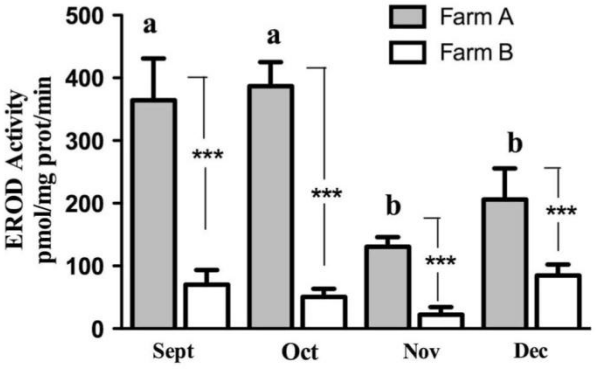

Fig. 4 (EROD activity in *O. mykiss* livers in farm A (*grey*) and farm B (*white*). Values are mean ± SEM ($n = 8$). *Asterisks* denote significant differences between farms at a given sampling time are indicated (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). Significant differences among samplings for the same fish farm are indicated by *lowercase letters* ($p < 0.05$)

that purpose, farm B, which had exhibited low EROD values, was considered the control cohort. In the first two samplings (September and October) *cyp1a* mRNA levels in farm A were 14 and 31 times greater, respectively, than in farm B ($p < 0.001$). In the following samplings (November and December), detected levels were considerably lower being only 2.5 times greater than in controls (Fig. 5a). *Ahr2* mRNA was significantly overexpressed in farm A with respect to farm B in October ($p < 0.001$), November ($p < 0.01$), and December ($p < 0.001$) showing mRNA levels between 1.8 and 2.4 times greater than that in the control fish (Fig. 5b). As a consequence, *Ahr2* mRNA expression levels in farm A in October were significantly ($p < 0.05$) greater than those in September.

Expression of cyp3a in Trout Liver

Hepatic *cyp3a* gene expression was also assessed in this study. In all sampling times, relative expression was greater in farm A compared with control fish. However, a different trend to that observed for *cyp1a* and *ahr2* was found. Although in the first two samplings, *cyp3a* was overexpressed approximately 5 times compared with the control ($p < 0.01$), the maximum induction was reached in November being 8 times greater than that in control fish ($p < 0.001$). One month later, in December, relative *cyp3a* gene expression was only 2.5 greater than that in farm B (Fig. 5c). For fish farm A, significantly ($p < 0.05$) greater values of *cyp3a* expression were only observed in November compared with December.

Expression of er and vtg in Trout Liver

To study whether the increased enzymatic activities were causing antiestrogenic effects, *er* and *vtg* gene expression was also assessed. In the first two samplings, relative *er* expression in farm A was similar to that found in the control (farm B). Only in the third and fourth samplings (November and December) was a significant ($p < 0.05$) increase in the expression of *er* observed in farm A with respect to the preceding months in the same farm and also with respect to farm B (Fig. 5d). For *vtg* gene expression, no difference across samplings or between studied (farm A) and reference (farm B) fish were found (data not shown).

Discussion

Sediments have been recognized as a major sink for pollutants in aquatic ecosystems. Thus, they have been widely

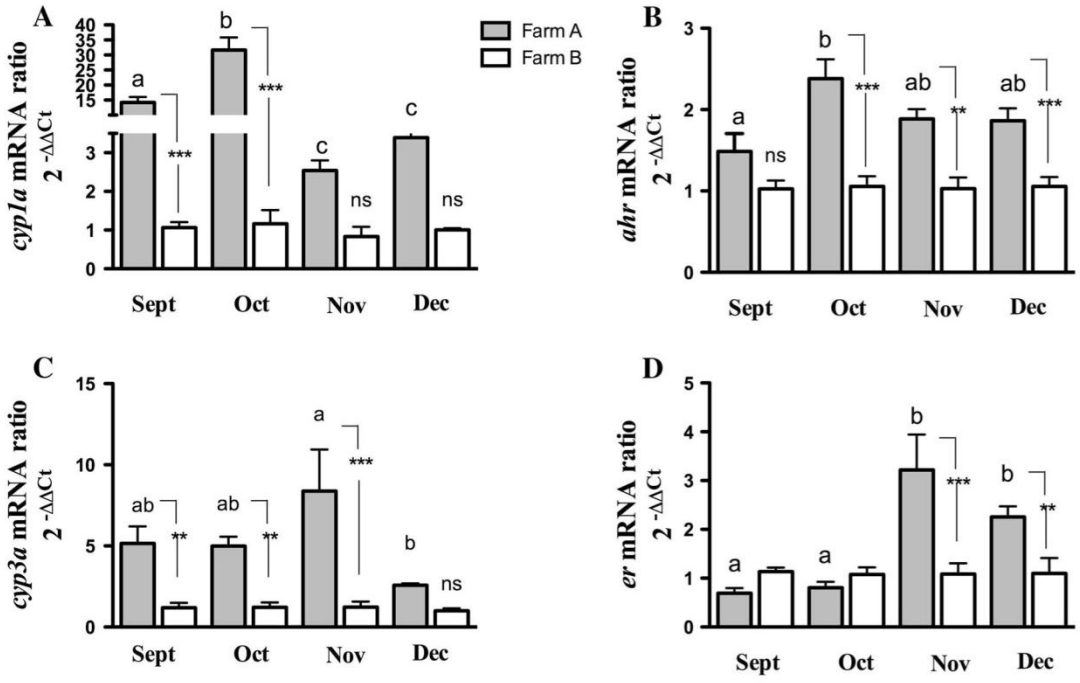

Fig. 5 *cyp1a* (**a**), *ahr2* (**b**), *cyp3a* (**c**), and *er* (**d**) mRNA expression in livers of *O. mykiss* determined by RT-qPCR. The results show the mRNA ratio defined as fold change in expression compared with farm B. Values are mean ± SEM ($n = 8$). Significant differences between farms at a given sampling time are indicated (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ [*NS* no statistically differences between farms]). Significant differences among samplings for the same fish farm are indicated by *lower-case letters* ($p < 0.05$)

monitored using chemical analyses (Pan et al. 2014; Duong et al. 2014; Pozo et al. 2014), biological approaches (Wang et al. 2014a; Huerta Buitrago et al. 2013) and combined methodologies (Qiao et al. 2006; Valdehita et al. 2012; Williams et al. 2014; Lei et al. 2014).

Taking into account their cost-effectiveness and easiness to use, in vitro cell bioassays have been commonly applied (Qiao et al. 2006; Lei et al. 2014) in monitoring studies. On some occasions, additionally, this approach sheds light on mechanisms of toxic action of environmental samples, which can be of high importance depending on the required information. Here, we assessed the presence of Ahr agonists in sediments with the gonadal cell line RTG-2. All sediments but one provoked an induction of EROD activity directly dependent on the sediment quantity to which the cells were exposed. This permitted us to calculate EC_{50} values for sediments and ßNF equivalents and thus to rank the sites according to the values obtained. Normally, TCDD is used as a reference for calculating TEQs from the empirically observed inductions (e.g., EROD) (Wang et al. 2014a; Qiao et al. 2006). However, considering that TCDD causes neurological and hepatotoxic effects (Pelclová et al. 2006) and that it is a known carcinogen in humans (National Toxicology Program 2011), in this study, we preferred to use the Ahr agonist ßNF as an alternative. ßNF is much less toxic than TCDD, thus facilitating its use and handling in the laboratories. In addition, inductions of Ahr-dependent responses caused by TCDD are on some occasions extremely strong, thus making difficult the comparison with soft responses caused by environmental samples with low TEQs content. This comparison becomes easier when ßNF is used as a reference, which makes this chemical the option chosen by different investigators (Babin et al. 2005; Noguerol et al. 2006; Boronat et al. 2009; Heinrich et al. 2014). Particular circumstances also favor the use of ßNF. For instance, when tests are performed in plastic plates, TCDD bioavailability is considerably decreased, presumably due to its hydrophobicity; therefore, the use of ßNF can be a suitable alternative (Miller 1999).

The calculated EC_{50} values for each sediment sample ranged between 0.89 and 5.42 mg sediment/ml, which are 3–30 times lower (therefore indicating a greater potency) than those reported in a previous work in which sediment samples from the entry channels of a fish farm were analyzed using the same methodology (Valdehita et al. 2012). ßNF-equivalents ranged from 0.49 to 4.09 ng Eq ßNF/mg sediment. These results, in the range of nanograms per milligram, are comparable with those previously reported in other works (Boronat et al. 2009; Puy-Azurmendi et al. 2010). For instance, Boronat et al. (2009), assessing Ahr agonistic activity in samples from high mountain lakes using a yeast reporter system, found values ranging from 0.035 to 1.5 ng Eq ßNF/mg sediment.

Screening chemical analyses performed with GC×GC–TOF–MS in the same sediment samples used in the bioassays detected the presence of a wide variety of substances commonly found in household wastewater, thus indicating the existence of low anthropogenic pressure. For instance, the musks AHTN and HHCB were ubiquitously present.

Quantification analysis also showed the presence of PAHs at concentrations (0.018–0.45 ng/g dw) similar to that reported previously in low populated areas (Quesada-Garcia et al. 2013; Valdehita et al. 2012). Total toxic equivalents (TEQs) were derived from the chemical analyses for each sample. They ranged between 0.16 (SS1) and 2.17 (SS2) pg TEQ/g dw, which is well lower than the safe sediment value of 20 pg TEQ/g dw (Evers et al. 1996).

Correlation analysis between ßNF-equivalents (derived from bioassay) and TEQ (obtained in chemical analyses) showed a positive association between both types of values. A good agreement between bioassay and analytical data has been reported previously (Qiao et al. 2006; Louiz et al. 2010). In addition, the slope of the adjusted line ($m = 0.281$) presented a greater value of ßNF-equivalents with respect to the TEQ values. Interestingly, although a good correlation ($r = 0.840$; $p < 0.05$) between ßNF-equivalents and TEQ existed, none of the tested PAHs (fluoranthene, anthracene, pyrene, and naphthalene) were able to induce EROD activity in RTG-2 at the applied doses either alone or in combination. These results are in agreement with previous studies in which the selected PAHs failed to induce in vitro EROD activity in the rainbow trout liver cell line RTL-W1 and also in primary hepatocytes (Bolsc et al. 1999; Behrens et al. 2001).

The three PCPs assayed (triclosan, HHCB, and AHTN) did not induce EROD in RTG-2 cells. To our knowledge, this is the first report assessing in vitro EROD induction of triclosan and HHCB using a piscine cell line. Contradictory data exist regarding the ability of triclosan to induce EROD activity in mammals (Jinno et al. 1997; Hanioka et al. 1996; Zorrilla et al. 2009). For instance, Hanioka et al. (1996) reported competitive inhibition of EROD activity in male Wistar rats treated with triclosan, whereas Jinno et al. (1997) reported increased activity in exposed rat hepatocytes. In fish, few studies have assessed this point, but contradictory results have also been found, probably due to a variety of factors including the different exposure concentrations, species tested, etc. Ishibashi et al. (2004) reported that triclosan does not induce EROD in female medaka. In contrast, Liang et al. (2013) found increased *cyp1a* mRNA levels and EROD activity in swordtail fish (*Xiphophorus helleri*). In agreement with our results, previous works have also shown the inability of HHCB to induce Ahr-dependent responses. For instance, European seabass (*Dicentrarchus labrax*) intraperitoneally injected

with HHCB showed no change in EROD activity (Fernandes et al. 2013); in addition, it does not activate human Ahr in vitro (Schreurs et al. 2005).

Even though the presence of AHTN has been commonly reported in environmental samples (Chase et al. 2012; Hu et al. 2011; Peck and Hornbuckle 2004), the literature concerning its ability to interact with Cyp1a is limited to a few reports (Schnell et al. 2009; Della Torre et al. 2011; Randelli et al. 2011). AHTN decreased EROD activity in carp microsomes when tested at a concentration of 100 μM (Schnell et al. 2009), but it had no effect in the enzymatic activity in the fish cell line PHLC-1 (Della Torre et al. 2011).

Despite the correlation between ßNF-equivalents and TEQcal ($r = 0.\ 840$, $p < 0.05$), inductions provoked by sediments were not explained by the analyzed compounds. This means that the observed toxic charges depend not only on the detected substances but also on the activity of contaminants present in undetectable concentrations and on the interactions (additive, synergistic, antagonistic) among them.

When applying chemical analysis to environmental samples, only chemicals for which standards and techniques are available are usually identified and quantified (Wang et al. 2014a). In addition, some chemicals, such as PAHs, usually do not appear alone in sediments but rather as mixtures of hundreds of related compounds with different properties and toxicity (Neff et al. 2005). In these complex mixtures, synergistic, agonistic, and antagonistic effects among substances pass unnoticed if only chemical data are available, thus probably leading to an underestimation of risks (Wang et al. 2014a). As an alternative, the use of in vitro bioassays has emerged as a rapid, inexpensive, and powerful tool to characterize samples and to obtain data that could be relevant for ecotoxicological hazard assessment (Wang et al. 2014a; Vincze et al. 2014). Although whole-animal models are able to integrate a number of environmental variables (pollution, temperature, photoperiod, etc.) and evidently present a greater ecological relevance than cell cultures, the current tendency to favour the "3Rs principle" (i.e., reduction, replacement, refinement) has favored the use of cell lines. In any case, these systems show some advantages that make them the tool of choice for particular analyses. First, they allow rapid and sensitive high-throughput screening, thus increasing the number of samples and substances tested in each experiment. Second, they provide mechanistic information on the toxic mode of action, which in environmental toxicology is of particular importance. In addition, cultured cells lack the complexity of organisms with sophisticated barriers and detoxification systems; therefore, particular interactions with cellular receptors that could potentially be harmful can be better characterized.

To determine whether fish are affected by the low levels of contaminants found in the river, a monthly sampling campaign was performed in the subsequent months. According to the literature, basal EROD activity in trout microsomes ranges from 4 to 100 pmol/mg/min (Brammell et al. 2010; Jonsson et al. 2006; Gourley and Kennedy 2009). The values detected in farm A in September, October, and December were 367, 387, and 206 pmol/mg/min, respectively, evidencing induced EROD activity. Fish sampled at the same time in another farm (named B) exhibited much lower values ranging from 22 to 85 pmol/mg/min. These inductions reported in farm A are comparable with those obtained in previous studies with juvenile rainbow trout (Quesada-Garcia et al. 2013; Valdehita et al. 2012). Although one reasonable origin of such inductions could be the low input of contaminants as evidenced by chemical analyses and in vitro *assays*, indeed the causes may be multiple. Another possible reason could be the pellets used to feed the animals. However, the feed used her was a commercial one commonly used in aquaculture and subjected to previous analyses, which proved that it did not induce EROD activity (data not shown). During the samplings, no veterinary drugs were administered to the animals, thus ruling out this possibility. Because confinement stress has been associated with an increase of EROD activity (Quabius et al. 2002), it is possible that differences in fish density between both farms could be related to differences observed in EROD measurements. Unfortunately, although we do not have exact data about fish density in the studied farms, fish farm A (devoted to the production of rainbow trout for river restocking with sport fishing purposes) showed the lowest density. Therefore, confinement stress does not appear to be the cause of the greater EROD levels observed in this farm. A fourth possibility could be the presence of substances of natural origin, such as humic substances or wood products, which are commonly present in freshwater ecosystems and are able to induce EROD activity (Matsuo et al. 2006). However, even in this case, the induction of detoxification activities is probably deviating energy from other relevant functions, such as growth, reproduction, or immunity, and the value of the present study relies on the ability of in vitro systems and farmed fish to evidence such inductions.

This deviation of energy could be related to the decreased HI observed in farm A with respect to farm B at all sampling times. Moreover, these values found in farm A are also lower than the value of 1 commonly reported for rainbow trout (De Francesco et al. 2004; Kumar et al. 2011). It has been proposed that the HI could be an useful measure of the general condition of fish in environmental studies (Facey et al. 2005). However, because liver plays a key role in detoxification, greater HI has been often associated with greater levels of pollution (Al-Ghais 2013;

Facey et al. 2005). Nevertheless, the opposite situation (i.e., lower HI in polluted environments compared with reference sites) has also been reported (Hauser-Davis et al. 2012; Traven et al. 2013). The causes of such inconsistencies are complex. First, exposure to some pollutant, such as PCBs may lead to increased HI (Leatherland et al. 1979), whereas exposure to others (e.g., paraquat or some heavy metals) may provoke the opposite effect (Akerman et al. 2003; Sindhe and Kulkarni 2004). In addition, increased HI may be also due to greater fat storage (Facey et al. 2005). Here, greater HI values are observed in fish also showing greater GI, thus indicating that these differences among farms may be also due to increased vitellogenin production (Andersson et al. 2007). Therefore, although it is a possibility, the complexity of factors affecting HI prevents us from ascertaining whether the decreased values observed in farm A are indeed caused by a deviation of energy due to greater EROD activity.

In the months in which EROD activity was highest (September and October), cytochrome induction was also observed at the transcriptional level with *cyp1a* mRNA levels highly overexpressed (between 14- and 31-fold compared with the external cohort [farm B]). In general, *cyp1a* mRNA and EROD activity have shown a positive association (Valdehita et al. 2012; Quirós et al. 2007), but the complex relationship between induction kinetics and mRNA and protein turnover (George et al. 2004) makes necessary the assessment of both end points at the same time.

Contrary to Cyp1a, which is used extensively as a biomarker of exposure (Brammell et al. 2010; Quirós et al. 2007; Jarque et al. 2010), less work has been performed on Cyp3a induction in fish. Here, relative *cyp3a* fold change ranged between 2.6 and 8.4. These results are comparable with that obtained in hornyhead turbot (*Pleuronichthys verticalis*) sampled at two polluted sites (Baker et al. 2009) where *cyp3a* mRNA levels were highly upregulated. Moreover, inductions ranging from 3- to 5-fold were obtained in trout hepatocytes exposed to oil sands process-affected water extracts (Gagne et al. 2012), which are known to contain a mixture of PAHs and other organic compounds (Rowland et al. 2011).

Although some have found a positive association between *cyp1a* and *cyp3a* (Gagne et al. 2012) in this study, no parallel trend was found. This could indicate that the observed inductions are provoked by different substances (or mixtures of compounds), which is in agreement with previous studies (Della Torre et al. 2010; Quesada-Garcia et al. 2013).

Conclusions

In the present study, analytical chemistry and biological monitoring techniques were combined to assess the possible presence of trace levels of contaminants in a river theoretically not subjected to anthropogenic pressure. Sediment samples induced EROD activity in vitro in the trout gonadal cell line RTG-2, and chemical analyses in the same samples showed the ubiquitous presence of low concentrations of a wide variety of substances including PAHs. Although a high correlation was observed between the TEQs derived from chemical analysis and ßNF-equivalents calculated from the in vitro-induced EROD activity, the detected PAHs and PCPs failed to provoke a response in the RTG-2 cells, thus indicating that other substances different from these detected by chemical analyses were present in the sediments and were responsible for the observed effects. Therefore, chemical analyses alone are not enough to predict pollutant effects on biota, and the combination of biological and chemical methods is advisable. In addition, trout from a fish farm located in the same river exhibited high levels of EROD activity and upregulation of genes related with detoxification responses (*cyp1a* and *cyp3a*), thus corroborating the presence of low levels of contaminants that can have subtle effects on biota, at least by deviating energy or resources from processes such as growth, reproduction, immune function, and detoxification activities. These results also demonstrate the suitability of the using of fish farms as sentinels for detecting the presence of trace levels of contaminants at a catchment scale, which can be a useful tool in ecological risk assessment.

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PAPER II:

USE OF FISH FARMS TO ASSESS RIVER CONTAMINATION: COMBINING BIOMARKER RESPONSES, ACTIVE BIOMONITORING AND CHEMICAL ANALYSES.

RESUMEN

En este estudio se evaluó el posible efecto de niveles traza de contaminantes en peces por medio de una combinación de técnicas: biomarcadores, biomonitorización activa y análisis químicos.En estudios ambientales, los citocromos P4501A (Cyp1A) y Cyp3A así como sus correspondientes actividades enzimáticas (7-ethoxyresorufin-O-deethylasq, EROD, and benzyloxy-4-[trifluoromethyl]-coumarin-O-debenzyloxylasa, BFCOD, respectivamente) se utilizan como biomarcadores para evidenciar la exposición a contaminantes. En una piscifactoria de trucha arcoiris (*Oncorhynchus mykiss*) rutinariamente muestreada para obtener valores de referencia de las citadas actividades enzimáticas, se observó un fuerte y puntual incremento en las mismas a finales de 2011. Con el objetivo de aclarar las causas de la inducción, se siguió un proceso de biomonitorización activa transfiriendo algunos peces a una piscifactoría control y observando sus respuestas en aguas limpias. Tras 7 días en la piscifactoría control, la actividad EROD se redujo en un 80% mientras que la BFCOD también se redujo tras 15 días. Asimismo se observó una reducción (no significativa) en los niveles de ARN mensajero de *cyp1a* y *cyp3a.* Para determinar la presencia de contaminantes, se tomaron muestras de sedimento y de agua del río que alimenta la piscifactoría y se analizaron con un sistema de GC × GC–TOF–MS. El estudio reflejó una débil entrada de contaminantes en el área monitoreada, la cual está localizada lejos de industrias o ciudades densamente pobladas. Niveles traza de hidrocarburos poliaromáticos (PAHs) y productos de higiene personal (HHCB y triclosan) se detectaron en los sedimentos, en concentraciones desde 0.01 a 38 ng/g peso seco y en muestras de agua en concentraciones desde 4 a 441 ng/L. La aproximación seguida en este estudio demuestra su utilidad como técnica de biomonitorización para la detección temprana de contaminantes traza.

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Use of fish farms to assess river contamination: Combining biomarker responses, active biomonitoring, and chemical analysis

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ABSTRACT

Here we addressed the possible effects of trace levels of contaminants on fish by means of a combination of biomarker responses, active biomonitoring (ABM), and chemical analysis. In environmental studies, cytochromes P4501A (Cyp1A) and Cyp3A and related enzyme activities (7-ethoxyresorufin-*O*-deethylase, EROD, and benzyloxy-4-[trifluoromethyl]-coumarin-*O*-debenzyloxylase, BFCOD, respectively) are commonly used as biomarkers for evidencing exposure to a variety of contaminants. In a rainbow trout (*Oncorhynchus mykiss*) fish farm that is routinely sampled to obtain references regarding normal levels of such enzyme activities in freshwater fish, we observed a strong and punctual increase in these activities at the end of 2011. In order to shed light on the causes of this induction, we transferred some fish to a fish farm with controlled conditions and examined them using an active biomonitoring (ABM) approach. EROD activity showed a decrease of 80% from the original values after 7 days in the control farm, while BFCOD activity was also reduced after 15 days. Although not significant, a decrease in *cyp1A* and *cyp3A* mRNA levels was also observed. To determine the presence of pollutants, water and sediment samples from the river feeding the fish farm were analyzed by two-dimensional gas chromatography–time-of-flight mass spectrometry (GC × GC–TOF–MS). The screening study reflected a weak inflow of pollutants in the monitored area, which is located far from any industrial activity or densely populated cities. Trace levels of polyaromatic hydrocarbons (PAHs) and personal care products (the polycyclic musk fragrance HHCB, and triclosan) were detected in sediments, at concentrations ranging from 0.01 to 38 ng/g dry weight, and in water from 4 to 441 ng/L. The approach followed in this study proved useful as a biomonitoring technique for the early detection of trace contaminants.

1. Introduction

Organisms in aquatic ecosystems are usually exposed to mixtures of xenobiotics in low concentrations with interactions that hinder the prediction of the deleterious effects on organisms when information is obtained exclusively from chemical analyses (Regoli et al., 2004). In this regard, the development of complementary monitoring methods is a priority.

Biomarkers are defined as biochemical, physiological or histological changes that are indicative of exposure to or effects of xenobiotics at the organismal and suborganismal level (Mayer et al., 1992). Some of the most commonly used biomarkers (reviewed in Celander, 2011; Peakall and Walker, 1994; Schlenk et al., 2008) are related to the cytochromes P450 (Cyps).

Cyp P450 enzymes are involved in reactions related to the biotransformation of many endogenous (steroids, bile acids, etc.) and exogenous (including pollutants and drugs) substances. Among them, Cyp1A plays a key role in the biotransformation of a wide variety of xenobiotics and as a consequence, it has been widely used as a biomarker of environmental pollution in aquatic ecosystems (Bucheli and Fent, 1995; Whyte et al., 2000). Induction of Cyp1A occurs after ligand-activation of the aryl hydrocarbon receptor (AhR). This receptor is typically, but not exclusively, activated by planar, polycyclic and aromatic compounds including polyaromatic hydrocarbons (PAHs), dioxins, and polychlorinated biphenyls

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(PCBs) (Denison et al., 2002). However, there is increasing evidence that activation of AhR is also triggered by other compounds, including some drugs and pesticides (Fernández-Cruz et al., 2011; Casado et al., 2006).

Also, Cyp P4503A contributes to detoxification processes, mainly of drugs. *Cyp3A* expression in mammals is regulated by the pregnane X receptor (PXR), whose promiscuity ensures an efficient clearance of structurally diverse chemicals, allowing the degradation of many lipophilic compounds (reviewed in Schlenk et al., 2008).

Cyp1A and Cyp3A have been used as biomarkers in aquatic species studying variations in their expression or function at the transcriptional, protein and enzyme levels (Della Torre et al., 2010; Sturve et al., 2006). Cyp1A enzymatic activity has been commonly measured as ethoxyresorufin-*O*-deethylase (EROD) (Whyte et al., 2000), while for Cyp3A one of the most frequently measured enzyme activities is benzyloxy-4-[trifluoromethyl]-coumarin-*O*-debenzyloxylase (BFCOD) (Hasselberg et al., 2008).

Biomarker methodologies have previously been combined with active biomonitoring (ABM) (He et al., 2011; Wepener et al., 2005), which involves the transplantation of organisms from one place to another to compare their responses (i.e. biochemical, physiological, community, etc.) (De Kock and Kramer, 1994). In general, organisms are moved from clean to polluted sites (He et al., 2011; Smolders et al., 2002), although the inverse procedure can be applied to demonstrate the recovery of organisms (Sutton et al., 2004). ABM approaches have been used with e.g. bivalves, algae and fish (Rotter et al., 2011; Tsangaris et al., 2011; Oikari, 2006). For the latter, ABM involves the use of cages (Birungi et al., 2007; Wepener et al., 2005). However, the maintenance of fish in reduced spaces can commonly cause additional stress, which may alter the results of the study. As an alternative, the use of fish in farms that receive waters from the rivers or lakes of interest has recently emerged (Valdehita et al., 2012).

To identify the contaminants to which organisms are exposed, mass spectrometry-based analysis, such as triple quadrupole (QqQ) and ion trap (IT) mass spectrometry, are the tools of choice, as they show high selectivity and detection limits down to ppt levels. These techniques offer key advantages in the identification and quantification of target compounds (Martínez-Bueno et al., 2007; Petrovic et al., 2005), but they need to operate by selecting ions (SIM), in MS/MS mode or by selecting reaction monitoring (SRM) to enhance selectivity, therefore limiting the number of compounds that can be analyzed. Consequently, many compounds and transformation products evade monitoring attempts. Over the past few years, and as a result of growing interest in non-targeted analysis, time-of-flight (TOF) mass analyzers have been increasingly used for the analysis of non-target and unknown compounds (Fernández-Alba, 2012).

In previous studies addressing EROD induction in fish, we routinely sampled a rainbow trout (*Oncorhynchus mykiss*) farm located in an isolated area, far from industrial activity or human exposure. In December 2011, EROD activity showed a sharp increase without any apparent reason. In this regard, the main objective of this study was to determine whether the strong induction observed was caused by the presence of certain contaminants. Combining different methodologies such as biomarker detection, ABM, and chemical analysis was essential for answering this question.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Sigma–Aldrich (CA, USA) unless otherwise noted. Tris–HCl, sucrose, KCl were purchased from Panreac Quimica S.L.U. (Zaragoza, Spain). The iScript RT-PCR kit with SYBR Green was from BIO-RAD (CA, USA). Analytical-grade methanol and sodium chloride (purity, 99.5%) were supplied by J.T. Baker (Deventer, Holland). Analytical-grade water was purchased from Fluka (Buchs, Switzerland).

2.2. Fish sampling and transplantation experiments

Routine samplings were performed on a monthly basis in a rainbow trout farm (A) located in the Autonomous Community of Castile-La Mancha, in an isolated area, far from any industrial activity and situated downstream of a village of less than 100 inhabitants. The farm is fed with water from a small river that flows parallel to the facility. From the entry channel, the water is distributed to the different tanks. The exit waters are collected in another channel, which flows to a decantation tank, where the solids are separated, and the water is recycled back to the river.

At the time of sampling, water conditions (i.e. oxygen saturation, temperature and pH) were measured. At each sampling, eight female juvenile rainbow trout (animals pertained to an all-female cohort) weighing approximately 200 g were anesthetized with 100 mg/L tricaine methane sulfonate (MS222) and killed by cervical dislocation. The animal fork length (±1 mm) and mass (±1 g) were recorded and liver and gonads weighed. Hepato-somatic and gonado-somatic indexes (HI and GI) were calculated following Gómez et al. (1999). Liver samples were immediately frozen in liquid nitrogen and stored at −80 °C until enzyme activity or gene transcription measurements.

In December 2011, the fish on this farm showed a three-fold unexpected increase in EROD activity, reaching a maximum in February 2012. Immediately after the detection of this peak, we implemented an ABM approach. With this purpose, several specimens from this farm (A) were transferred to a second fish farm (B), which served as a control. Farm B received clean water from a 100 m deep well, and was therefore not affected by anthropogenic pressure or contamination of any kind. After 7 days of acclimatization in farm B, eight of the transferred animals were killed and enzyme activities were determined. The transplantation experiment was repeated some months later (September 2012). In this case, 24 fish were transferred to farm B, and periodic samplings of eight of them were done at days 7, 15 and 30.

2.3. Sampling of fish feed, sediments and water

In all sampling campaigns, two sediment samples were taken from the entry channel of farm A for chemical analysis. In addition, in September 2012, sediment and water samples (two samples of each type per sampling site) were taken 4000 m (site sampling 1 (SS1)) and 2000 m (SS2) upstream of the farm. These points were located before (SS1) and right after (SS2) the only village present in the area, and were chosen to determine whether the little anthropogenic pressure present could account for the elevated EROD and BFCOD activities. Water samples (1 L) were collected in clean amber glass bottles pre-rinsed with MilliQ water. Sediment samples were taken from the top layer river bed (0–7 cm), where pollutants are expected to accumulate. In addition, fish feed samples were taken to analyze the possible presence of pollutants. Once in the laboratory, all the samples were stored at 4 °C prior to analysis, which was performed within 24 h to avoid degradation.

2.4. EROD and BFCOD activities in trout liver

The microsomal fraction from liver was isolated as described by Valdehita et al. (2012). EROD activity was then measured following Burke and Mayer (1974) as described elsewhere (Valdehita et al., 2012).

Table 1
Primers for quantitative PCR analysis of gene expression.

Target gene	Primers sequence (5′...3′)	Product size (bp)	Reference
B-actin	Sense: CATCACCATCGGCAACGA Antisense: GATGTCCACGTCACACTTCATGA	137	Valdehita et al. (2012)
Cyp3A	Sense: TCTACCCTGCTGAGCGGAA Antisense: ACAGTGGGTTGAACAGGTCG	198	This study
Cyp1A	Sense: TCAACTTACCTCTGCTGGAAGC Antisense: GATGAACGGCAGGAAGGA	68	Rees and Li (2004)
Vtg	Sense: GCTGCCCTTGATGAGAACGAC Antisense: TCCCAAGACAACCTCAGACGA	158	Tilton et al. (2006)

BFCOD activity was measured following the method described by Thibaut et al. (2006) optimized for the rainbow trout microsome-enriched fraction. Briefly, the assay consisted of incubating 5 μl of the microsome-enriched fraction with 250 μM of 7′-benzyloxy-4-(trifluioromethyl coumarin) (BFC) and 200 μM of NADPH in potassium phosphate buffer 200 mM pH 7.4 with bovine serum albumin (1.6 mg/mL). Fluorescence was measured at excitation and emissions wavelengths of 409 and 530 nm, respectively.

In both cases, the protein content was measured using the fluorescamine assay (Kennedy et al., 1995; Navas and Segner, 2000).

Each assay was run three independent times for each individual sample, and each time was run in triplicate.

2.5. RNA extraction from rainbow trout liver and single-step reverse transcriptase quantitative PCR (RT-qPCR)

Total RNA was extracted from 0.1 mg of liver using TRI® reagent, following the manufacturer's instructions. RT-qPCR analysis was carried out with 90 ng of RNA for each sample, using an iScript RT-PCR kit with SYBR Green in a one-step protocol, following the manufacturer's instructions. The thermal cycling conditions were 10 min at 50 °C for the reverse transcription, 5 min at 95 °C to inactivate the reverse transcriptase, and 35 cycles at 95 °C for 10 s and at 58 °C for 30 s. All PCR reactions were performed in a Line-Gene K System (BIOER Technology, Hangzhou, China) with the specific primers for *cyp1A*, *cyp3A* and *vitellogenin* (Table 1). The relative quantification of the mRNA of the target gene was normalized to β-*actin* (Valdehita et al., 2012). Results were represented as *C*t values, where *C*t is defined as the threshold cycle number at which a product is first detected by fluorescence. β-*Actin* mRNA expression was constant among the fish cohorts, with average values for *C*t β-*actin* mRNA of 15.52 ± 0.29. Thus, β-*actin* was considered an appropriate reference gene for normalization in this study. Relative quantification was measured using the comparative Ct method, also referred to as $2^{-\Delta\Delta Ct}$, representing the amount of target normalized to the endogenous control (β-*actin*) and relative to the mean value of an external set of trout (fish from a third independent farm, farm C), where $\Delta\Delta Ct = (Ct_{target} - Ct_{\beta\text{-}actin})$ fish farm A − $(Ct_{target} - Ct_{\beta\text{-}actin})$ fish farm C. The fold change in relative expression was then determined by calculating $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001). All RT-qPCR reactions were performed in triplicate.

2.6. Sample treatment and GC × GC–TOF–MS analysis

Samples of sediments and fish feed were directly extracted by solid liquid extraction (SLE) with methanol, as described by Valdehita et al. (2012) and analyzed by means of stir-bar sorptive extraction (SBSE), followed by comprehensive two-dimensional gas chromatography (GC × GC–TOF–MS) (Gómez et al., 2011). The GC × GC–TOF–MS system consisted of an Agilent 7890A (Agilent Technologies, Palo Alto, CA, USA) gas chromatograph, equipped with a secondary oven to fit the secondary column, and a quad-jets modulator (two cold jets and two hot jets). The first column was a Rtx-5 (10 m × 0.18 mm i.d., 0.2 μm) from Restek (Bellefonte, PA, USA) and the second column a Rxi-17 (1 m × 0.1 mm i.d., 0.10 μm), also from Restek. High purity helium at a flow rate of 1.5 mL/min was used as the carrier gas. The chromatographic conditions were as follows: the first-dimension column oven temperature program began at 70 °C for 3 min, then increased to 150 °C at a rate of 30 °C/min, followed by a 5 °C/min ramp to 200 °C, and finally at a rate of 15 °C/min to 285 °C, and held at this temperature for 5 min – the total analysis time was 27.17 min. The second-dimension column oven temperature began at 15 °C, then 20 °C, and finally 30 °C higher than the corresponding first-dimension column oven temperature with the same rate and hold time. The MS system was a Pegasus 4D TOF from LECO Corporation (St. Joseph, MI, USA). Electron impact (EI) mass spectra in full-scan mode were obtained at 70 eV. The temperature of the transfer line and the ion source were set at 280 and 250 °C, respectively. The TOF–MS was run in the range of 50–450 *m*/*z* at an acquisition rate of 100 spectra/s with a detector voltage of 1460 V. Auto-tuning for TOF–MS optimization was done prior to each optimization test sequence.

Instrument control and data processing were conducted with the Leco ChromaTOF (version 4.24) software. Data processing included automatic peak finding using MS deconvolution and spectral searches against the NIST 2008 and Wiley mass spectral libraries. All the validation studies of the performance of the method in wastewater and river water samples have been described previously (Gómez et al., 2011).

2.7. Statistics

The results are expressed as the mean ($n = 8$) ± standard error of the mean (SEM). All the statistical analyses were performed with Sigma Plot 11.0 (San Rafael, CA, USA). Data distribution, verified with Kolmogorov–Smirnov test, was not normal, thus imposing the use of non-parametric tests. The differences between sampling months were assessed by a Kruskal–Wallis one-way ANOVA on Ranks followed by post hoc Dunn's multiple comparison test. The differences in the enzyme activities and mRNA expression of some genes after transfer from farm A to farm B were assessed by a Kruskal–Wallis one-way ANOVA on Ranks followed by post hoc Dunnett's multiple comparison versus control test. Significant differences were at $p < 0.05$.

3. Results

3.1. Water conditions and hepato- and Gonado-somatic indexes (HI and GI)

Water pH values were similar in all samplings, ranging from 7 to 8.4. Oxygen saturation in the water was always 100% air saturation. Water temperatures in farm A showed the normal seasonal variation. The minimum temperature was recorded in January (7.7 °C) while the maximum was registered in September (16.9 °C). Regarding the months in which transplantation experiments were performed in February 2012, the temperature in both farms was

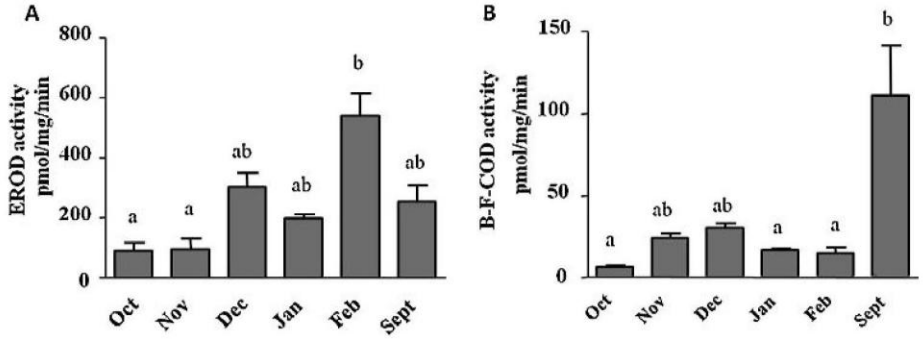

Fig. 1. (A) 7-Ethoxyresorufin-*O*-deethylase (EROD) and (B) benzyloxy-4-[trifluoromethyl]-coumarin-*O*-debenzyloxylase (BFCOD) activity in *Oncorhynchus mykiss* livers sampled at several time points. Values are mean ± SEM ($n = 8$). Significant differences between samplings are indicated by lower case letters ($p < 0.05$).

8.2 °C and in September 2012, temperatures in fish farms A and B were 16.9 °C and 19.0 °C, respectively.

No differences between samplings were observed for the HI or the GI, the mean values being 0.912 ± 0.182 and 0.330 ± 0.044, respectively. The low GI values confirmed that the fish sampled were juveniles.

3.2. EROD and BFCOD activities

In the first two samplings (October and November 2011), EROD activity values were 91 ± 26 pmol/mg protein/min and 92 ± 34 pmol/mg protein/min, respectively (Fig. 1A). In the subsequent sampling, December 2011, EROD activity levels showed a considerable, although not significant, increase, reaching values of 300 pmol/mg protein/min. This enzyme activity remained elevated in January 2012 (200 pmol/mg protein/min) and continued increasing until reaching a maximum in February 2012 (541 ± 74 pmol/mg/min), this value being significantly ($p < 0.05$) higher than those observed in October and November.

Regarding BFCOD activity, no changes (values ranging from 7 ± 1 pmol/mg protein/min in October to 26 ± 5 pmol/mg protein/min in December) were detected between October 2011 and February 2012 (Fig. 1B).

Six months later, (September 2012), we performed an additional sampling. EROD activity continued to be high (254 ± 56), although lower than in February; however, BFCOD reached maximal levels (102 ± 28 pmol/mg protein/min).

3.3. mRNA levels of cytochromes P4501A and P4503A and of vitellogenin in trout liver

The mRNA expression of *cyp1A* increased ($p < 0.05$) in November 2011, reaching levels four times higher than those detected in the previous month (Fig. 2A). In the following samplings (December and January), a decreasing trend suggested recovery of *cyp1A* mRNA near basal levels. In February, mRNA of *cyp1A* was again overexpressed, reaching about seven times the values registered in October. Finally, in the additional sampling (September 2012), *cyp1A* mRNA expression showed values similar to those detected in October.

Regarding *cyp3A*, the mRNA expression of this gene in November, December and January was lower than in October 2011 (Fig. 2B). The only significant ($p < 0.05$) difference was obtained between October and January. In February and September 2012, mRNA expression exhibited levels similar to those found at the beginning of the samplings.

To study whether the increased enzyme activities affected the mRNA expression of estrogen-dependent genes, mRNA levels of *vitellogenin*, were also determined by RT-qPCR. Values were low and no significant changes were observed between samplings (data not shown), thereby confirming that fish were juveniles and discarding any potential estrogenic effect associated with the increased EROD and BFCOD activities.

3.4. Active biomonitoring (ABM) studies

In the ABM, at first 16 fish were transferred in February 2012 to the control farm B and maintained there for one week. After only 7 days, EROD activity dropped to less than 25% of the original values, practically recovering the initial levels recorded in October and November 2011 (135 ± 15 pmol/mg protein/min) (Fig. 3A). At the same time, BFCOD activity also decreased by half (7 ± 2 pmol/mg/min), recovering the values observed in October (Fig. 3B). In September of the second year, another ABM experiment showed a decrease in EROD activity to 20% of the original values (final values 48 ± 12 pmol/mg/min) after 7 days. Additional

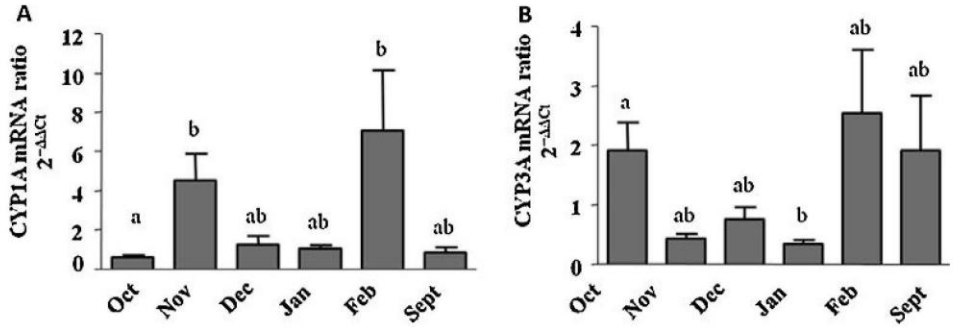

Fig. 2. (A) *cyp1A* and (B) *cyp3A* mRNA expression in liver of *Oncorhynchus mykiss* determined by RT-qPCR. The results show the mRNA ratio defined as fold change in expression compared to an external control. Values are mean ± SEM ($n = 8$). Significant differences between samplings are indicated by lower case letters ($p < 0.05$).

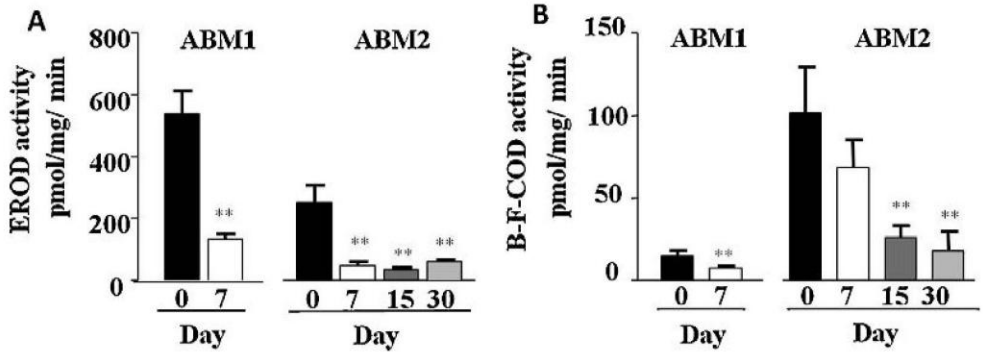

Fig. 3. (A) 7-Ethoxyresorufin-*O*-deethylase (EROD) and (B) benzyloxy-4-[trifluoromethyl]-coumarin-*O*-debenzyloxylase (BFCOD) activity in *Oncorhynchus mykiss* livers from the two active biomonitoring (ABM) experiments. The first experiment (ABM1) took place in February 2012 and lasted 7 days; the second experiment (ABM2) took place in September 2012 and lasted 30 days. Values are mean ± SEM. ($n = 8$). Double asterisk denote significant differences being $p < 0.01$.

maintenance in the control farm for 15 and 30 days did not lead to further decreases in EROD activity. Regarding BFCOD activity, again, after only 7 days in the control farm, Cyp3A-related activity was reduced by almost half (60 ± 17 pmol/mg protein/min). Additional maintenance for 15 and 30 days produced a further significant ($p < 0.05$) decrease in this enzyme activity (reaching 26 ± 7 pmol/mg protein/min and 18 ± 12 pmol/mg protein/min, respectively) with respect to the initial values.

In addition, a decrease in *cyp1A* and *cyp3A* mRNA levels was also observable after fish transplantation to the control farm (Fig. 4).

3.5. Chemical characterization by GC × GC–TOF–MS analysis

Fig. 5 shows the contour plot with all the compounds detected by the GC × GC–TOF–MS analysis in sediment and river water samples collected upstream (4000 m from the fish farm) and downstream (2000 m from the fish farm) of the village. Over 3100 and 3800 compounds were detected, respectively, in the GC × GC chromatogram for these samples by a data processing method, where *S*/*N* was set at 30. Chromatographic peaks are marked with black dots and, depending on the intensity, signals are shown in a color range, from yellow, indicating low intensity, to red, indicating high intensity. Non-targets were characterized by screening through an entire "Peak Table" obtained after data processing, which contains the library search results showing, among other parameters, the name of the compounds and mass spectral similarity factors. A minimum similarity factor of 600 over a maximum match factor of 999 was set as identification criteria. Using this technique, one of the identified compounds was HHCB (1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethyl-cyclopenta-[g]-2-benzopyrane; trade name: Galaxolide®). Fig. 5A presents a 3D contour plot zoom of the area where HHCB elutes. The concentrations of HHCB detected in this study ranged from 37 to 440 ng/L in waters and reached up to 2.6 ng/g dry w. in sediments.

Chemical analyses also evidenced the ubiquitous presence of PAHs in the study area, with concentrations ranging from 4 to 39 ng/L in water and from 0.01 to 38.40 ng/g dry w. in sediments (Tables 2 and 3). Fig. 5B shows a 3D contour plot zoom of the area where PAHs elute. The high similarity match factor, reflected by good quality mass spectra, allowed the identification of phenanthrene, naphthalene, fluoranthene and pyrene (Fig. 5B).

Regarding the differences between samplings, which could explain the observed inductions, pyrene and fluoranthene were found at a considerably higher concentration in February 2012. In this case, the concentration of pyrene was between 100 and 275 times higher than in September, October, and November 2011. In addition, the concentration of fluoranthene was 11 times higher than in November and September and 200 times higher than in October, when basal levels of *cyp1A* mRNA and EROD activity were measured. Another personal care product punctually detected in sediments was triclosan (5-chloro-2-(2,4-dichlorophenoxy)phenol) in concentrations ranging from 0.23 to 0.30 ng/g dry w.

In fish feed, only residues of the PAHs phenanthrene and naphthalene were detected at trace concentrations (0.36 and 3.75 ng/g dry w., respectively).

4. Discussion

Here we addressed whether the strong induction of EROD and BFCOD activities observed in the livers of rainbow trout were

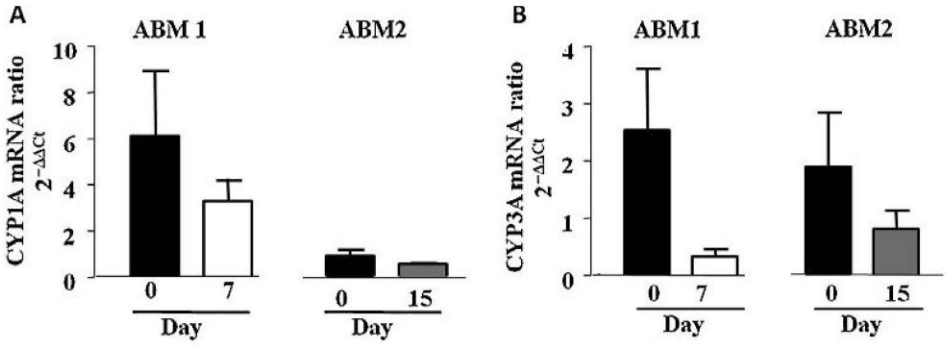

Fig. 4. (A) *cyp1A* and (B) *cyp3A* mRNA expression in livers of *Oncorhynchus mykiss* determined by RT-qPCR in the two active biomonitoring (ABM) experiments. The first experiment (ABM1) took place in February 2012 and lasted 7 days; the second experiment (ABM2) took place in September 2012 and lasted 30 days. The results show the mRNA ratio defined as fold change in expression compared to an external control. Values are mean ± SEM ($n = 8$). No significant changes between sampling dates were found.

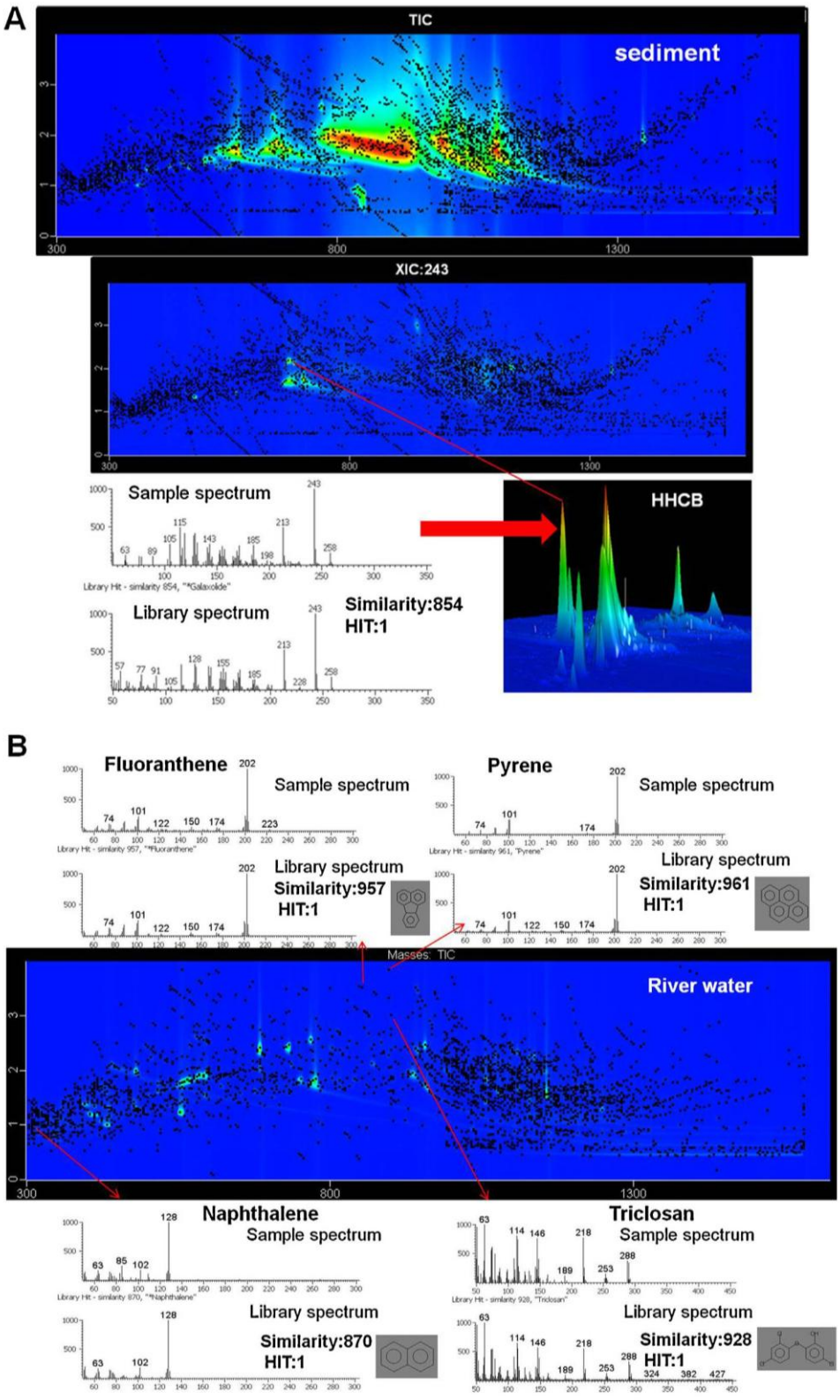

Fig. 5. GC × GC–TOF–MS contour plots and peak table of sediment (A) and water river (B) samples collected 4000 (SS1) and 2000 m (SS2) upstream of the fish farm, respectively. Identification of non-target compounds: HHCB (A, upper panel) and PAHs (phenanthrene, naphthalene, fluoranthene and pyrene; lower panel – B) with the corresponding library and reference spectrum.

Table 2
Concentrations of substances detected by GC × GC–TOF–MS in sediments (0–7 cm) of the entry channel in fish farm A (ng/g dw sediment).

	October 2011	November 2011	February 2012	September 2012
Phenanthrene	0.26	0.44	0.39	0.24
Naphthalene	6.98	2.93	1.64	1.32
Fluoranthene	0.02	0.36	4.02	0.34
Pyrene	0.18	0.41	38.40	0.14
Triclosan	0	0.23	0	0.30
HHCB	1.17	2.16	2.69	2.64

caused by trace levels of contaminants. These fish were from a fish farm (named farm A) located in an isolated area, far from any human pressure or industrial activity that could cause such inductions. According to the literature, basal EROD activity levels in rainbow trout microsomes range from 30 to 100 pmol/mg/min (Brammell et al., 2010; Gourley and Kennedy, 2009). The values detected initially, in October and November, can therefore be considered physiological while values measured in the following months can be seen as induced.

Water temperatures influence EROD activity, although controversial results exist on whether decreases in temperature enhances or decreases this activity (Abrahamson et al., 2008; Andersson and Koivusaari, 1985; Lyons et al., 2011). However in the present study, differences in temperatures between subsequent samplings were only of 1–2 °C maximum meanwhile in the cited studies changes in EROD activity levels occurred at temperatures differing in more than 5 °C. This means that water temperature changes do not explain the variations observed in EROD activity. In addition, enzyme activities dropped significantly after fish were transferred to the reference fish farm, even though temperatures were similar. Variations in EROD activity could also be associated with physiological seasonal changes. Previous reports in other fish species have shown an increase in EROD activity in spring-summer probably associated with higher biological activity than in the winter (Koenig and Solé, 2012; Nahrgang et al., 2010). However, in the present work the maximal EROD activity was observed in winter (February). If the Cyp1A induction was not caused by water temperature or seasonal variations, our results support the notion that the fish were exposed to contaminants. In fact, the EROD induction recorded in December and February is comparable with that reported in juvenile rainbow trout receiving 5.04 mg/L of the fungicide propiconazole (Li et al., 2013).

In contrast to EROD activity, which has been extensively used as a biomarker of environmental pollution (Jolly et al., 2012; Locatello et al., 2009; Valdehita et al., 2012), BFCOD has been applied for this purpose in only a few studies (Hasselberg et al., 2008; Hegelund et al., 2004; Wassmur et al., 2010). The activity in rainbow trout has been reported to range from 2 to 20 pmol/mg/min (Hasselberg et al., 2008; Hegelund et al., 2004; Wassmur et al., 2010) meaning that the BFCOD activity recorded between October and February can be considered basal. Interestingly, during the same period, Cyp1A activity showed an increasing trend, implying that the substances responsible for EROD induction do not trigger Cyp3A activity. In the additional sampling performed six months later (September 2012) BFCOD reached maximal levels. Descriptions of the induction of BFCOD activity in fish are scarce. To the best of our knowledge, only Della Torre et al. (2010) have assessed the response of BFCOD activity in a field study. Specifically, they studied the effect of PAH contamination on red mullet (*Mullus barbatus*). In this case, BFCOD activity was three times higher in the polluted area than the reference site. Whether the strong Cyp3A induction observed in our study in September is attributable to the presence of the aforementioned pollutants in the river remains to be clarified.

At the transcriptional level, the *cyp1A* induction observed in February 2012 (7-fold compared to October 2011) is comparable to that reported in juvenile trout fed a high-dose mixture of PAHs (7.82 μg PAH mixture/g fish/day) for 50 days (Bravo et al., 2011). This observation is perhaps indicative of the response in the present study. We found no parallel trend when comparing *cyp1A* or *cyp3A* mRNA with EROD or BFCOD activities, respectively, in trout livers. While many studies on fish describe a high association between the two endpoints (mRNA expression and associated enzyme activity of the gene) (Meyer et al., 2002; Quiros et al., 2007; Valdehita et al., 2012), others report a low association or non-correlation (Della Torre et al., 2010; Kammann et al., 2008). Regarding Cyp3A, the discrepant findings between enzymatic and transcriptional level are in accordance with previous studies, which also found a lack of correlation between BFCOD activity and *cyp3A* mRNA expression. In the particular case of Cyp3A it is necessary to take into account that although the substrate used for making the measurements, BFC, is a Cyp3A-specific substrate in mammals, in fish it can be metabolized by other Cyps, including Cyp1A (Scornaienchi, 2009). However, our data support the contrary, as if BFC were metabolized by Cyp1A, EROD and BFCOD would have shown similar induction patterns. These differences between mRNA expression of a gene and enzyme activities of the gene product raise the question to which endpoint is more appropriate as a biomarker. Taking into account that such differences are probably the consequence of the complex relationship between induction kinetics and mRNA and protein turnover (George et al., 2004) that we begin to understand nowadays, the most suitable approach in field studies would be their complementary application.

After the 3-fold and unexplained increase in EROD activity in February, we followed an ABM approach in which several rainbow

Table 3
Concentrations of substances detected by GC × GC–TOF–MS in sediments (ng/g dw sediment) and water samples (ng/L) collected along the river feeding the fish farm A in September 2012.

	SS1[a]		SS2[a]		Entry channel	
	Sediment	Water	Sediment	Water	Sediment	Water
Phenanthrene	0.01	9	0.11	4	0.24	6
Naphthalene	1.17	39	0.23	12	1.32	33
Fluoranthene	–	–	0.14	–	0.34	–
Pyrene	–	–	0.03	–	0.14	–
Triclosan	–	–	–	–	0.30	–
HHCB	2.36	37	2.27	50	2.64	441

–, <LOD (limit of detection in water, in ng/L): phenantrene, 0.01; naphthalene, 0.02; fluoranthene, 0.05; pyrene, 0.03; triclosan, 0.06 and HHCB, 0.02.
[a] SS1 and SS2 are located 4000 and 2000 m upstream of the fish farm, respectively.

trout from farm A were transferred to a clean farm (Farm B). The strong decrease in both enzyme activities and mRNA levels after transferring the fish to clean water is consistent with the notion that the fish had been exposed to contaminants. Thus interruption of this exposure allowed the catabolization or elimination of the responsible compounds (Brammell et al., 2010), leading to the reduction or even disappearance of the detoxification responses. Indeed, juvenile rainbow trout exposed to various AhR agonists and then maintained in clean water for 14 days showed a progressive recovery of basal levels (Jönsson et al., 2006).

In the framework of the present study, we consider that the differences observed in EROD and BFCOD activities or in *cyp1A* and *cyp3A* mRNA levels between sampling times are related to variations in the concentrations of substances able to induce the related detoxification response. This would be consistent with the possibility of isolated spillages of pollutants caused by domestic or agricultural activities, which are common in the study area.

Trying to identify the compounds responsible of the observed inductions, chemical analyses were performed using a GC × GC–TOF–MS system. The coupling of GC × GC with TOF–MS has the advantage of a full range of non-skewed mass spectral information for all peaks with full-range sensitivity, thus allowing us to screen and highlight non-target compounds which might be relevant for further impact assessment (Gómez et al., 2011). Using a minimum similarity factor of 600, this analytical method allowed detecting the ubiquitous presence of HHCB, which is a polycyclic musk widely used as a fragrance ingredient in personal care products and in domestic cleaning products. This compound modulates mRNA expression levels of *cyp3A40* in male medaka (*Oryzias latipes*) (Yamauchi et al., 2008) and is a strong inhibitor of Cyp3A-catalyzed activities ($IC_{50} = 68 \pm 12\ \mu M$) (Schnell et al., 2009). On the other hand, Schreurs et al. (2004) reported that HHCB does not activate human AhR *in vitro*, thus indicating that Cyp1A is not induced. Given this observation, the induction of EROD activity detected in our study cannot be associated with the presence of this substance in the water and sediments analyzed.

Another personal care product detected in sediment at some points was triclosan, a broad spectrum antimicrobial used as antiseptic, disinfectant or preservative in many household products. Triclosan shares some structural similarities with polychlorobiphenyls and as such is a selective inhibitor of the glucuronidation and sulfonation of phenolic xenobiotics (Wang et al., 2004). Results of studies on triclosan-induced activation of Cyps in fish are contradictory. For instance, Ishibashi et al. (2004) reported that triclosan does not induce EROD activity in female adult medaka (*O. latipes*). However, a recent study with swordtail fish (*Xiphophorus helleri*) found that both *cyp1A* and *cyp3* mRNA levels, as well as the associated enzyme activities (EROD and erythromycin N-demethylase, respectively) were increased after exposure to concentrations of this compound ranging from 0.002 to 1.25 mg/L (Liang et al., 2013). Given the limited data in the literature and the discrepancies between studies, the inductions that we observed cannot be directly associated with the presence of triclosan. Further research is required to clarify the potential of triclosan as an inductor of cytochromes.

Chemical analyses also evidenced the presence of trace concentrations of PAHs, which are prominent inducers of Cyp1A (Navas and Segner, 1998). Pyrene and fluoranthene were found at a considerably higher concentration in February 2012. In this case, the concentration of pyrene was between 100 and 275 times higher than in September, October, and November 2011. In addition, the concentration of fluoranthene was 11 times higher than in November and September and 200 times higher than in October. Thus the presence of one or a combination of pyrene and fluoranthene in a much higher concentration may underlie the strong induction of EROD activity and mRNA level of *cyp1A* observed in February 2012. It must be taken into account that there exists a plethora of substances, probably present at concentrations that are not individually detectable with the current analytical techniques, but that are also contributing to the observed inductions. The interactions among these substances imply not only simple additive but also synergistic and antagonistic effects that we are now beginning to understand (reviewed by Celander, 2011). Thus, although the detection of these two PAHs does not alone explain the observed induction, it could be indicative of the presence of substances with similar features that went undetected by the chemical methods used, but that also favored the observed inductions. In any case, taking all this together, the simultaneous presence of these two compounds in a considerable high concentration could serve as an indicator of an episode of punctual contamination.

Lastly, the finding of residues of phenanthrene and naphthalene at trace concentrations in fish feeds could also explain the inductions. However, during the transplantation experiments, fish were fed the same pellets, but cytochrome induction continued to be reduced so that fish feed cannot be directly related with the inductions detected.

5. Conclusions

Combining biomarker responses, ABM, and chemical analyses by means of GC × GC–TOF–MS, the methodology described here has proven useful in detecting trace levels of contaminants in water courses and evidencing their influence on biota. GC × GC–TOF–MS, is a powerful technique that allow the screening of low concentrations of contaminants of concern in a single-run analysis. Furthermore, we have shown the capacity of biomarkers to provide early warning signs of diffuse contamination of anthropogenic origin. The ABM studies performed here confirmed that the fish were exposed to bioactive factors and, given the high sensitivity of Cyp1A and Cyp3A, that these pollutants were present. The detection of increased concentrations of particular PAHs, simultaneously with the highest induction of EROD and BFCOD, may indicate that this substance class contributed to the increased biomarker activities.

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CHAPTER II:
Presence of endocrine activity in commercial fish feeds.

PAPER III:

ASSESSMENT OF ESTROGENIC AND THYROGENIC ACTIVITIES IN FISH FEEDS

RESUMEN

El correcto funcionamiento del sistema endocrino es esencial para el desarrollo y reproducción de los animales. Aquellas sustancias que interfieren con su homeostasis se denominan disruptores endocrinos (DEs) y pueden suponer una amenaza para la salud de los organismos. Uno de los mecanismos de disrupción endocrina más estudiado en los últimos años se refiere a interferencias con el receptor de estrógenos (ER). Sin embargo, el eje tiroideo, que en peces juega un papel crítico en una variedad de funciones biológicas, ha sido mucho menos estudiado. En acuicultura, los piensos pueden ser una fuente de hormonas o contaminantes persistentes, que a su vez actúan como disruptores endocrinos. El objetivo principal de este estudio era evaluar la posible carga estrogénica y tirogénica de 32 piensos comerciales. Para evaluar la estrogenicidad, se desarrolló y validó un nuevo ensayo de gen informante (usando el receptor de estrógenos alfa, ERa de lubina). La potential disrupción a nivel tiroideo, fue evaluada con una línea celular transfectada de manera estable con el gen informante de luciferasa bajo el control del receptor de hormonas tiroideas alfa (THRa) de origen aviar. Los resultados mostraron que 11 y 18 de los 32 piensos evaluados fueron capaces de activar el sbERa y el avTHRa1, respectivamente. El presente estudio es pionero en demostrar actividad tirogénica en piensos de peces comerciales y ampliamente usados en acuicultura. Dado que mantener la homeostasis en el sistema endocrino es crucial para el apropiado desarrollo y reproducción de los peces, cualquer actividad estrogénica o tirogénica provocada por los piensos debe ser tenida en cuenta por su potencial impacto en los peces cultivados.

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Assessment of estrogenic and thyrogenic activities in fish feeds

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ABSTRACT

Normal functioning of the endocrine system is essential for the proper development and reproduction of animals. Substances interfering with its homeostasis are called endocrine disruptors (EDs) and may represent a risk for the health of the organism. One of the mechanisms of endocrine disruption that has attracted great attention in recent years concerns alterations in the normal functioning of the estrogen receptor (ER), but far less attention has been paid to those substances interfering with the thyroid axis, which, in fish, plays several critical roles in a variety of biological functions. In aquaculture, feedstuffs can be a source of hormones or persistent pollutants which act as potential EDs. In this study, the main purpose was to assess the possible estrogenic and thyrogenic activities of 32 commercial fish feeds. For the assessment of estrogenicity, a new estrogen receptor specific reporter gene assay using sea bass ERα (sbER α) was developed and validated. Potential thyroidal disruption was screened with a cell line permanently transfected with luciferase as reporter gene under the control of avian thyroid receptor α (THRα). The results obtained showed that 11 and 18 out of 32 assayed feeds were able to activate the sbERα or the avTHRα1, respectively. The present study is pioneer in demonstrating thyrogenic activity in fish diets commercially available and widely used in aquaculture. Given that maintaining the homeostasis in the endocrine system is critical for the proper development and reproduction of fish, any estrogenic or thyrogenic activity caused by the feedstuffs should be taken into account with regards to its potential impact on farmed fish.

1. Introduction

Endocrine disruptors (EDs), as defined by the World Health Organization (WHO), are "exogenous substances or mixtures that alter function(s) of the endocrine system and consequently cause adverse health effect in an intact organism, its progeny, or (sub) populations" (WHO/IPCS, 2002). The impact of EDs is of particular concern in teleost fish since these animals are exposed to waterborne contaminants during their whole life span. Intensive fish culture makes feedstuffs an alternative vehicle for the incorporation of persistent EDs. The principal potential sources of feed contamination are ingredients of animal origin, and fish aquaculture diets are not an exception (Mantovani et al., 2009; Pelissero and Sumpter, 1992). Long-term sustainability of intensive aquaculture requires the replacement of fish meal and fish oils in aquafeeds by vegetable equivalents (Drew et al., 2007; Gatlin et al., 2007; Glencross et al., 2007). However, plant meals used as substitutes also contain substantial quantities of EDs that negatively affect fish physiology (Beresford et al., 2011; Matsumoto et al., 2004; Pelissero and Sumpter, 1992). Soybean meal is the main source of vegetable protein present in animal diets, although a large number of studies have shown that a high dietary percentage of soybean meal may result in decreased growth and reproductive changes in fish (Drew et al., 2007; Pelissero and Sumpter, 1992). The poor growth rates exhibited by fish fed diets rich in soy flour have been attributed to the presence of estrogenic isoflavones, e.g. daidzein and genistein, in the bile of these fish (Kaushik et al., 1995). In fact, estrogenicity of commercial fish feeds has already been assessed using yeast estrogen-screen assays (Matsumoto et al., 2004) or in vivo experiments (Beresford et al., 2011).

Estrogenic substances can emulate the action of the endogenous estrogen via activation of the estrogen receptors ERs which work as ligand-activated transcription factors. Following agonist binding, the receptor undergoes a conformational change which enhances its affinity for DNA, where it interacts with specific sequences called estrogen responsive elements (ERE), inducing the expression of estrogen-dependent genes (Beato and Klug, 2000). These genes are mainly related to reproduction, differentiation and growth. However in teleosts,

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estrogens are involved in immune system regulation and several studies have related estrogen-like disruptors with immunosuppression (Milla et al., 2011). For all the above, anti/estrogenic compounds have received substantial attention in recent years (Hotchkiss et al., 2008).

Unfortunately, far less attention has been paid to the detection of substances that may disrupt the hypothalamus–pituitary–thyroid axis (HPT). The thyroid of fish secretes L-thyroxine (T4) into the circulation. T4 enters target cells, where it undergoes monodeiodination to 3,3′,5-triiodo-L-thyronine (T3). Thyroid hormones (THs) play critical roles in growth, metabolism and development in all vertebrates (Yen, 2001). But, in fish and amphibians, the thyroid axis also plays a key function in normal development and metamorphosis, larval stages being particularly sensitive to the disruption of the HPT axis (Blanton and Specker, 2007; Carr and Patiño, 2011). There is also evidence that TH may be involved in gonadal sex differentiation, probably via its action on aromatase activity (Mukhi et al., 2007), but also in the proliferation of Sertoli and Leidyg cells and, by extension, in the testis development and function (Matta et al., 2002).

In recent years, many chemicals have been suspected of acting as thyroid disruptors including some polychlorinated biphenyls (PCBs), tetrabromobisphenol A (TBBPA) and polybrominated diphenyl ethers (PBDEs) (Boas et al., 2006). These substances may compete with the endogenous hormones for binding to transport proteins (transthyretin) and/or to TH receptors (TR), acting as either agonists or antagonists and disrupting TH homeostasis (Boas et al., 2006; Kashiwagi et al., 2009). TRs, together with the steroid receptors, belong to the nuclear receptor family and act as ligand-dependent transcription factors which bind to a specific region of the DNA named TRE (thyroid hormone responsive element). Previous studies have found considerable levels of PCBs and dioxin-like substances in fish feeds (Berntssen et al., 2010), where they could mimic endogenous TH and potentially lead to thyroid disruption. To the best of our knowledge, the presence of thyroidal disruptors in fish diets has never been tested. The aim of this study was to simultaneously assess the potential estrogenic and thyrogenic activity of 32 commercial fish diets using hormone receptor-mediated reporter gene activation. For the assessment of estrogenicity, a new estrogen receptor specific reporter gene assay, using sea bass ERα (sbERα) was developed (Muriach et al., 2008). The assay was validated using 17β-estradiol (E2) analogs and ER antagonists and through the screening of sewage effluent samples, previously reported as containing considerable estrogen loads (Carbonell et al., 2011). Potential thyroid disruption was screened with a reporter gene under the control of avian (av) THRα1 (Jugan et al., 2007). We demonstrate that extracts from 11 of the 32 assayed fish diets activated sbERα, while, 18 diets activated avTHRα1.

2. Materials and methods

2.1. Chemicals

17β-Estradiol (≥98% purity), tamoxifen (≥99% purity), 17-α-estradiol (≥ 98% purity), 17-α-methyltestosterone (≥ 97% purity), 3′, 5-Triiodo-l-thyronine (T3, ≥98% purity), etnylenediaminetetraacetic acid (EDTA), sodium dodecyl sulfate (SDS), methanol (≥99.9% purity), tricineKOH, bovine serum albumina (BSA), $MgCl_2$, isobutylmethylxanthine (IBMX), dithiothreitol (DTT), ATP, Coenzyme A hydrate (CoA) and luciferin were purchased from Sigma-Aldrich (Madrid, Spain). Fetal bovine and horse serum (FBS and FHS), ultraglutamine, penicillin–streptomycin (10.000 U/ml), hygromycin, tripsin, geneticin, ultraglutamine 1, and cell culture Dulbecco's Minimal Essential Medium (DMEM) were obtained from Lonza (Barcelona, Spain). Phenol red-free DMEM was from PanBiotech (Zaragoza, Spain). The stock solutions of E2, 17-α-estradiol, tamoxifen and T3 were prepared in DMSO; 17α-methyltestosterone was dissolved in ethanol.

2.2. Extraction of EDs present in fish food

Thirty two commercially available fish feeds were tested for estrogenicity and thyroidal activity. The extraction of estrogenic and thyrogenic substances was carried out with methanol as previously described (Cerdá-Reverter et al., 1996; Matsumoto et al., 2004; Rodríguez et al., 2000) with minor modifications: 0.5 g of each diet were sonicated in 2.5 ml of methanol using Vibra Cell™ ultrasonic probe (Sonic & Materials Inc., Newtown, CT, USA) at 18 kHz in three pulses of 15 s (70% amplitude). Homogenates were then centrifuged at 1700×*g* for 10 min. Supernatants were vacuum-dried and resuspended in 300 μl of methanol. The extracts were maintained at −20 °C until their assessment in the cellular assays.

Recovery tests were designed to evaluate the efficiency of the extraction method. Briefly, 300 μl of a solution of, either 100 μM E2 or 100 nM T3 in methanol, were added to 0.5 g of a diet showing no estrogenic or thyrogenic activity and extracted as above. The spike solution was applied directly onto the grinded feedstuff allowing the interaction of the hormones with the matrix for 30 min. The resulting extracts were named as E2-feed and T3-feed. In order to ensure the proper recovery of the hormones in the methanol and their stability during the extraction process, hormone solutions of E2 and T3 were submitted to the same extraction process. These extracts were named E2–MeOH, and T3–MeOH and contained a concentration of either 100 μM E2 or 100 nM T3, respectively.

Additionally, in a set of fish feeds, this process was performed using hexane as described by Ramos et al. (2004) in order to extract non-polar substances.

2.3. Generation of HER-LUC cell line

The HEK-293 cell line, stably expressing sbERα (Muriach et al., 2008) was cotransfected, in proportion 50:1, with a construct (ERE-TK-LUC) containing the luciferase gene under the control of tandem repetitions of the estrogen responsive element (ERE; Muriach et al., 2008) and a construct carrying resistance to puromycin (Muñoz et al., 2005). Cells were grown in 96-well plates and selected with DMEM (Invitrogen) containing 10% fetal bovine serum (Invitrogen), penicillin (100 units/ml), streptomycin (100 μg/ml) and puromycin (8 μg/ml; Invitrogen) in a humidified atmosphere of 5% CO_2 at 37 °C. Luciferase activity was tested after incubating resistant clones in 96-well plates (15,000 cells/well) with assay medium (DMEM medium + 0.1 mg/ml bovine serum albumin, BSA + 0.1 mM, IBMX) containing 10^{-6}–10^{-12} M estradiol. Forty eight hours post-treatment cells were washed twice with saline phosphate buffer, resuspended into 100 μl of reporter lysis buffer (Promega) and stored at −80 °C until luciferase activity determinations. Lysed cells were pelleted by centrifugation for 30 s at 15,000×*g*, and 20 μl of the supernatant was mixed with 200 μl of luciferin reagent (20 mM TricineKOH, pH 7.8, 0.1 mM EDTA, 8 mM $MgCl_2$, 33.3 mM DTT, 270 μM CoA, 530 μM ATP, 400 μM luciferin). The light emitted was measured in a luminometer (Junior, EG&G, Berthold). The most sensitive cell clone was named as HER-LUC and was selected for subsequent studies. To evaluate unspecific responses due to any basal luciferase transcription activity, HEK 293 cells were transiently transfected only with ERE-TK-LUC construct and exposed to equivalent E2 concentrations.

2.4. Cell culture

HER-LUC cells were grown in 75 cm^2 flasks under 5% CO_2 humidified atmosphere at 37 °C in DMEM supplemented with 10% FBS, 1% antibiotic mixture (Penicillin/Streptomycine) and 2% Ultraglutamine.

The PC-DR-LUC cell line derived from PC-12 cells stably expressing the avTRα1 (Muñoz et al., 1993) and the luciferase reporter gene (Jugan et al., 2007) was used to assess the thyroidal activity of the fish feeds. PC-DR-LUC cell line was a kind gift from Juan Bernal

(CSIC, Madrid) and prior to the assessment of the environmental samples, it was confirmed that the cells responded to T3 in a comparable way to that described in Jugan et al. (2007). EC50's of T3 were in the same range (0.18 nM in Jugan's study and 0.068 nM in the present work) and the maximal induction factors were similar (15.5 fold and 12 fold, respectively). Cells were grown in 75 cm^2 flasks under 5% CO_2 humidified atmosphere at 37 °C, in DMEM containing 4.5 g/l glucose, 15% serum (10% horse serum + 5% fetal calf serum), 1% antibiotic mixture (penicillin/streptomycin), 1 mM ultraglutamine, 0.8 mg/ml geneticin and 0.8 mg/ml hygromycin B. The cells were split weekly with 0.5% trypsin/0.02% EDTA.

2.5. Luciferase reporter gene assay optimization using HER-LUC

Cells were seeded into 96-well, white, opaque cell culture plates (Perkin Elmer, Groningen, The Netherlands) at a density of 25×10^4 cells per well in DMEM medium. The response of HER-LUC to E2 was evaluated in different culture medium conditions. First, experiments were focused on the study of the effects of FBS or phenol red on sbERα-mediated luciferase activation. Cells were grown in DMEM containing FBS or charcoal-treated FBS and, lastly, serum-free medium. The effect of phenol red was evaluated by using phenol red-free medium.

The response to different agonists and antagonists was evaluated by incubating HER-LUC cells with different concentrations of test compounds (17β-estradiol, 17α-estradiol and 17α-methyltestosterone) ranging from 10^{-9} to 10^{-3} M.

Similarly, to evaluate the possibility that the induction of luciferase could be mediated by a factor other than ER activation, HER-LUC cells were pretreated with the ER antagonist tamoxifen at concentrations ranging from 0.039 to 12.5 μM for 2 h. Then, cells were treated with E2 0.25 μM. After 24 h, the medium was discarded, and cell viability and luciferase activity were measured as described in the following section.

To assure the ability of the cell line to detect estrogenicity in field samples, water from sewage treatment work effluents was extracted as described by Fernández et al. (2010). Briefly, Solid Phase Extraction (SPE) cartridges (Oasis HLB cartridges 200 mg, Waters, Barcelona, Spain) were used. Cartridges were conditioned with 3 ml of methanol and 3 ml of MilliQ water. Then, 500 ml of each water sample was loaded in the cartridge that was washed with methanol. The cartridge was vacuum dried for 30 min and the retained compounds eluted with (2×) 4 ml of 10% methanol that was evaporated under a stream of nitrogen. Extracts were reconstituted in 300 μl of DMSO. Serial dilutions in the culture medium were prepared for the cell exposure (ranging from 0.12 to 8.3 ml effluent/ml medium).

2.6. Screening of possible EDs in fish feed

Cell lines (HER-LUC and PC-DR-LUC) were exposed to different concentrations of fish feed extracts equivalent to a range from 0.008 to 16.7 mg fish feed/ml for 24 h. Positive (1 μM of E2 or 1nM of T3) and negative (methanol 1%) controls were always added. Additionally, possible interferences or antagonistic effect were tested by pretreating the cells with the corresponding extract of fish feed during 3 h. Subsequently, cells were exposed to the hormone (either E2 or T3) at the EC50 concentration.

After incubation, cell viability was determined by measuring the cellular metabolic activity with the resazurin method (O'Brien et al., 2000). Given that resazurin does not interfere with the luminiscence assay (data not shown), the cell viability assays were performed prior to luciferase assays in the same plate. 5 μl of the resazurin dye solution (ToxKit8, Sigma-Aldrich, Madrid, Spain) was added to each well and plates were incubated at 37 °C and 5% CO_2 for 90 min. Fluorescence was then read with a Microplate-Reader (Tecan Genios Pro, Maenndorf, Switzerland) using 530 nm and 590 nm as excitation and emission wavelengths, respectively. As a positive control of cytotoxicity, 1 mM SDS was also included in each assay. Luciferase activity was then measured using a luciferase reporter gene assay kit (Biodetection Systems, Amsterdam, the Netherlands) according to the manufacturer's instructions with small modifications. Briefly, 90 μl or 120 μl of phosphate buffered saline (PBS, pH 7.5) for the HER-LUC or the PC-DR-LUC cell lines, respectively and 30 μl of the lysis buffer were added. After 15 min, 80 μl (HER-LUC) or 50 μl (PC-DR-LUC) of the luciferase reagent was added and bioluminescence was measured in the culture plates using a luminometer (MicroBeta Trilux, Perkin Elmer, USA).

2.7. Data and statistical analysis

The results are presented as mean ± standard error of the mean (SEM) of at least two independent experiments (between two and six independent experiments were carried out, depending on the test). All the statistical analyses were performed using GraphPad Prism version 5 (GraphPad Software, San Diego, CA, USA). The luciferase fold induction was expressed as the ratio between mean value of light units from exposed and non-exposed control wells. The normal distribution of data was verified with the Shapiro–Wilk test. Statistical significance of luciferase induction compared with its control was tested by one-way repeated measures analysis of variance (ANOVA) followed by Dunnet's test.

The estimation of the concentration–response function and the calculation of the EC_{50} (effective concentration 50, defined as the concentration causing a response 50% of the maximal response) were made by fitting the luminescence results to a regression equation for a sigmoid curve: $y = \max/[1 + (x/EC_{50})b] + \min$, where max is the maximal response observed, b is the slope of the curve and min is the minimal response. Relative transactivation activity (RTA) of each tested compound was calculated by normalizing its maximal luciferase fold induction with respect to that produced by 0.25 μM of E2 (set at 100%). Relative agonistic activities (RAA) allow chemicals to be ranked according to their potency for estrogen receptor activation and were calculated by dividing the EC_{50} value of the E2 by the EC_{50} value of the compound of interest. The RTA of each fish feed was calculated by normalizing its maximal luciferase fold induction with respect to that produced by the corresponding positive control (PC) (fish feed spiked with either 0.5 nM T3 or 0.25 μM E2, set at 100%). The lack of a complete dose–response curves in some of the fish feeds for either of the assays meant that the EC_{50} values could not be calculated in those cases.

3. Results

3.1. Characterization of HER-LUC cell assay

In order to establish the best conditions to perform the luciferase reporter gene assays with the HER-LUC cell line, the interferences caused by phenol red, FBS or hormones present in FBS were evaluated. Fig. 1 shows typical dose–response curves obtained by treating cells with increasing concentrations of E2 for 24 h under different culture conditions. In complete media (containing phenol red and 10% FBS), the maximal response was reached at a concentration of 0.25 μM E2 with $EC_{50} = 0.029$ μM. The results obtained with phenol red-free medium showed that E2 efficacy (maximal response) was approximately half that obtained with complete medium, indicating the activating effect of phenol red on ER. In this case, the EC_{50} value was 0.025 μM, pointing to a similar potency of stimulation as obtained with the complete medium. Serum deprivation during the treatment resulted in an E2 efficacy similar to that obtained with phenol red-free medium and an EC_{50} of 0.0069 μM. Although these conditions led to an increase in the potency of the assay, cells cultured without serum appeared extremely weak, and were easily detached and lost during exposure to

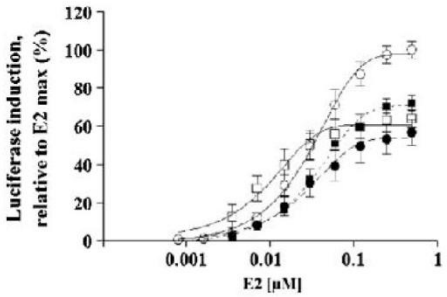

Fig. 1. Luciferase induction of HER-LUC cells after exposure to E2 in media containing phenol red and serum (○), media without phenol red (●), serum-free media (□) and phenol red-free and charcoal-treated serum media (■). The results are shown as% with respect to the maximal induction caused by E2 in complete media (considered 100%). Data points represent mean ± SEM of six independent experiments performed in duplicate.

feed extracts. Data from cells grown in phenol red-free media and charcoal-treated serum (to avoid the presence of hormones) resulted in the typical sigmoidal response curve. In these conditions, the EC_{50} was 0.032 μM and the maximum response was obtained at concentration of 0.25 μM. Thus, these were the conditions selected to carry out the assays.

Prior to the assessment of the fish feeds, the responsiveness of the new HER-LUC cell line to 17α-estradiol, 17β-estradiol, 17α-methyltestosterone and tamoxifen were evaluated. All the selected chemicals have been proposed by the Organization for Economic Co-operation and Development (OECD) as reference chemicals in in vitro estrogenicity studies (OECD Guideline 455, 2009). Fig. 2 shows dose-dependent response curves corresponding to the four chemicals. 17β-estradiol was the most potent and efficient estrogenic compound with $EC_{50} = 0.032$ μM and a maximal response caused by 0.25 μM, followed by 17α-estradiol, which was approximately 100 times less potent than 17β-estradiol ($EC_{50} = 2.854$ μM), and achieved its maximum induction (RTA 84.6%) at 80 μM. As expected, 17α-methyltestosterone was seen to be a much weaker agonist, with an EC_{50} of 380 μM. The maximum response of 17α-methyltestosterone was obtained at 950 μM (RTA of 15% compared with that of E2). Relative agonistic activities (RAA) varied within five orders of magnitude; 17α-estradiol had an RAA of 0.01, while in the case of 17α-methyltestosterone it was 8.53×10^{-5} (RAA E2 = 1). The antagonist tamoxifen inhibited the maximal response induced by E2 in a dose-dependent manner. The IC_{50} was 3.56 μM and concentrations of tamoxifen above 12.5 μM completely antagonized the E2-induced response. The exposure of cells to the same range of tamoxifen doses did not induce luciferase activity. The possible cytotoxicity of all compounds was evaluated by means of the resazurin method (O'Brien et al., 2000). None of the tested agonists or the antagonist tamoxifen was toxic.

When the cells were transfected only with ERE-TK-LUC construct in order to evaluate unspecific responses, the results showed a lack of any response to the E2 treatment.

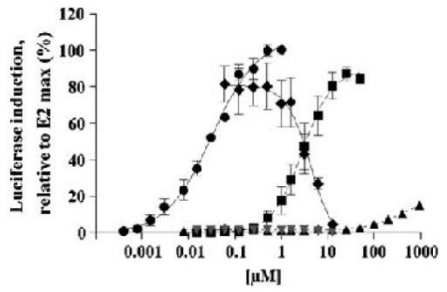

Fig. 2. Dose–response curves for the tested agonists, E2 (●); 17α-estradiol (■) and 17α-methyltestosterone (▲). The antagonist tamoxifen was tested both alone (▼) and coincubated with 0.25 μM E2 (♦). Data points represent mean ± SEM of at least three independent experiments performed in duplicate and are shown as% with respect to the maximal induction caused by E2.

3.2. Estrogenic activity of sewage water

In order to evaluate the screening capacity of the newly developed system with complex environmental samples, cells were also exposed to extracts of waste water effluents previously reported as containing considerable estrogen loads (Carbonell et al., 2011). Concentrations ranging from 0.12 to 8.3 ml effluent/ml medium were able to induce full dose–response curves (Fig. 3), indicating the suitability of the new cell line for the detection of estrogenicity. Interestingly, relative transcription activities (RTA) reached 100% of that produced by 0.25 μM of E2.

3.3. Evaluation of the efficiency of the extraction

Fig. 4 shows the typical dose-dependent response curves obtained after stimulation of HER-LUC (4A) or PC-DR-LUC (4B) cells with increasing doses of E2 and T3, respectively.

In HER-LUC cells, both E2 and E2 submitted to the extraction procedure (E2–MeOH) showed similar efficacy and potency (EC_{50} values of 0.032 and 0.036 μM, respectively), meaning that the extraction process itself does not affect the stability of the hormone and that the recovery was maximal with MeOH. When the fish feed was spiked with E2 and extracted, the potency of stimulation was similar ($EC_{50} = 0.051$ μM). However, the extraction efficiency was reduced to 66%. These results indicate that the added hormone might be strongly complexed with other substances present in the fish feed so that the extraction is not complete.

In PC-DR-LUC cells the EC_{50} values for T3 and T3–MeOH were 0.068 and 0.127 nM, respectively, these results show that T3–MeOH presented lower stimulation potency than intact T3, although the amplitude of the response was similar in both cases. In the dose–response curve belonging to T3-spiked feed, a lower efficacy was observed than in the T3 or in the T3–MeOH curves, 62% of the induction obtained with T3 being reached, with an $EC_{50} = 0.168$ nM.

To improve hormone recovery, a double extraction with methanol was performed, but this did not lead to an increase of the maximal signal (efficacy) in the curves obtained with the extracts of the spiked feed (data not shown). Taking into account that the complex matrix prevents higher recoveries, the dose–response curves obtained with the hormone-spiked feed extracts (E2-feed, T3-feed) were used as reference when assessing the estrogenicity or thyroid activity in the fish feeds.

3.4. Estrogenic activity of fish feed extracts

The potential estrogenicity of commercial fish feeds was tested in HER-LUC cells. Two types of extraction, using methanol and hexane, were assayed to detect polar and non-polar compounds, respectively. The equivalent concentrations of fish feed to which cells were exposed ranged from 0.008 to 16.7 mg fish feed/ml. Extracts obtained with hexane did not stimulate sbERα-induced luciferase activity (data not shown). Previously, cell viability was evaluated through resazurin transformation into the fluorescent resorufin, expressed as relative fluorescence units (RFU). Fish feed concentrations inducing a decrease in RFU ≥ 80% were considered cytotoxic and excluded from the analysis. In the three diets (F3, F9 and F31) extracted with methanol, the RFU was enhanced significantly compared with the control levels (Fig. 5).

As regards estrogenicity assessment, 11 of the 32 fish feeds significantly induced luciferase activity. Fig. 5 shows the induction of

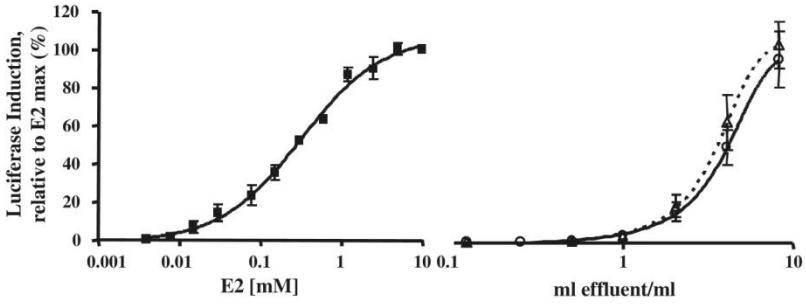

Fig. 3. Dose response curves of E2 (A) and two waste water effluents (B): effluent 1 (○) and effluent 2 (Δ). Data points represent mean ± SEM of three independent experiments performed in duplicate and are shown as% with respect to the maximal induction caused by E2.

luciferase activity in 6 representative samples at two consecutive concentrations, the maximum concentration inducing an effect and the preceding concentration. Higher concentrations than those shown in the figure did not give additional stimulation due to a cytotoxicity effect. Three feeds (F2, F7 and F9) were able to induce a two-fold increase in luciferase activity when tested at 1, 0.13 and 0.26 mg/ml respectively. In addition, F27 and F31 induced a weak significant increase in the luciferase activity of about 50% at a concentration of 0.015 and 0.06 mg/ml, respectively. F3 showed the highest estrogenic activity, inducing a three-fold increase when tested at a concentration of 16.7 mg/ml (EC_{50} = 9.57 mg/ml). Table 1 shows the maximum RTA of the 11 fish feeds with estrogenic activity. In this case, the RTA value is relative to that of the positive control (E2-feed). In ten of the tested diets, the RTA represented between 1.05 and 3.05% but the estrogenicity of the F3 feed was 6.05% of that obtained in the E2-feed.

Antiestrogenic effects were not observed. None of the tested extracts were able to produce any decline in the hormone induction and the response obtained from the co-incubation (feed extract plus hormone) corresponded to the effect provoked by the hormone alone, indicating the absence of interferences (data not shown).

3.5. Thyroidal activity of fish feed extracts

The potential disrupting effect at the TR level was also assessed in the same commercial fish diets (concentrations ranging from 0.008 to 16.7 mg fish feed/ml). Again, extracts done with hexane had no effect on the reporter activity (data not shown). For the methanol extracts, the cytotoxic criteria considered, (measured with the resazurin method) were the same described above. In this case, none of the diets provoked an increase in the fluorescence at any concentration. The thyroidal activity assessment shows that 18 out of 32 assayed fish feeds induced a significant luciferase activation when compared to the control levels. Increasing concentrations of the extracts resulted in increased induction of luminescence, with maximum responses observed at concentrations ranging from 4.1 to 16.7 mg fish feed/ml. 14 out of 18 positive diets were able to induce a full dose–response curve and thus, EC_{50} values could be derived (Table 1). All EC_{50} values were in the same order of magnitude ranging from 1.09 to 5.23 mg of fish feed/ml. The induction of luciferase activity in 12 representative feeds at two consecutive concentrations (the maximum concentration provoking the effect and the preceding concentration) is shown

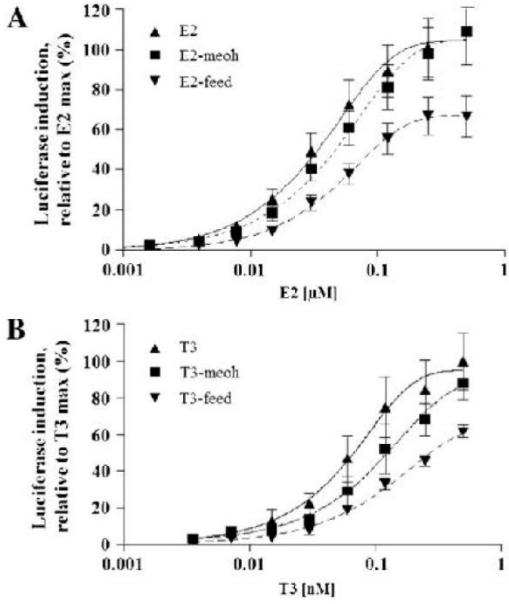

Fig. 4. Dose response curves in HER-LUC (A) and PC-DR-LUC cells (B), after exposure to their corresponding pure hormone, hormone extracted with methanol and spiked-fish feed. Data points represent mean ± SEM of five to nine independent experiments performed in duplicate. The results are shown as% relative to maximal induction of pure hormones, E2 (A) and T3 (B) (considered 100%).

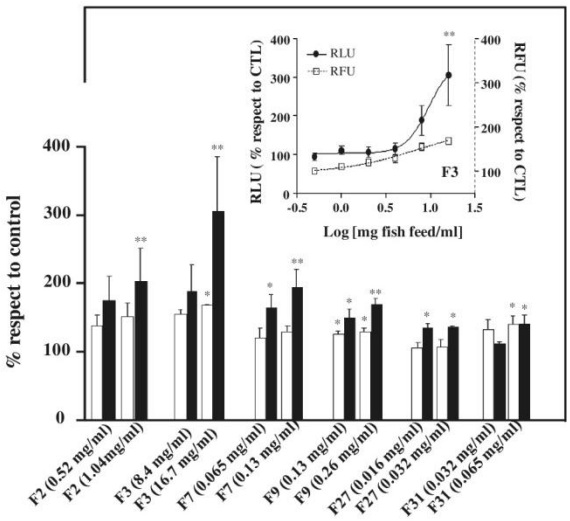

Fig. 5. Estrogenic activity represented as relative luminescence units (RLU, black) and cytotoxicity as relative fluorescence units (RFU, white) in HER-LUC cells. The data represented show the effect of the maximal concentration inducing the estrogenic effect and its immediately previous concentration. Columns represent the mean of at least two independent experiments ± SEM. The inset shows the full dose response curve belonging to one of the samples (F3). Asterisks show statistical significant differences with respect to corresponding (luminescence or fluorescence) controls (*: $p<0.05$; **: $p<0.01$).

Table 1
Maximal estrogenic/thyroidal activity of fish feeds determined by HER-LUC/PC-DR-LUC assay. Significance: *: $p<0.05$; **: $p<0.01$.

Fish feed sample	ER induction			TR induction		
	C Max	RTA	EC_{50} (mg feed/ml)	C Max	RTA	EC_{50} (mg feed/ml)
F1	-	-	-	4.2**	9.96 ± 2.60	1.31
F2	1.04**	3.05 ± 1.45	NDR	4.2*	11.82 ± 1.45	1.61
F3	16.7**	6.05 ± 2.30	9.57	4.2**	17.49 ± 2.08	2. 03
F4	-	-		8.4**	23.78 ± 2.18	3.00
F5	-	-		8.4**	14.55 ± 1.14	1.71
F6	-	-		8.4**	16.30 ± 2.37	1.40
F7	0.13**	2.78 ± 0.81	NDR	4.2**	24.24 ± 1.61	2.97
F8	-	-		4.2*	13.68 ± 1.50	1.76
F9	0.26**	2.04 ± 0.28	NDR		-	
F10	0.065**	1.38 ± 0.25	NDR		-	
F11	0.065**	2.25 ± 0.27	NDR	8.4**	29.19 ± 5.71	1.30
F12	0.065**	1. 41 ± 0.14	NDR	-	-	
F14	0.065*	1.19 ± 0.09	NDR	-	-	
F16	-	-		4.2**	23.06 ± 1.96	1.77
F17	-	-		4.2*	20.78 ± 1.01	1.70
F18	-	-		8.4**	22.69 ± 4.52	3.21
F19	0.065**	1.39 ± 0.39	NDR	8.4**	47.64 ± 5.68	1.09
F20	-	-		16.7**	35.46 ± 0.008	NDR
F21	-	-		16.7**	18.10 ± 5.95	NDR
F23	-	-		16.7*	13.48 ± 5.10	NDR
F27	0.0325*	1.05 ± 0.09	NDR	-	-	
F30	-			8.4*	18.34 ± 7.04	2.32
F31	0.065*	1.20 ± 0.39	NDR	-	-	
F32	-		-	16.7**	27.33 ± 10.93	5.23

C Max: Concentration inducing maximal effect (mg fish feed/ml).
RTA: Relative transactivation activity. Percentage of induction respect to maximal E2-feed or T3-feed response (0.25 μM E2 or 0.5 nM T3 respectively.
NDR: No dose response was observed impelling the calculation of EC_{50} value.

in Fig. 6. Higher concentrations than those represented in the figure resulted cytotoxic and did not provoke any increase in luminescence. The representative dose–response curves for F4 (6A) and F32 diets (6B) are shown as insets.

The RTAs are shown in Table 1. In 9 diets, RTAs were between 10 and 20% whereas 7 diets exhibited RTAs between 20 and 30%. Finally, diet F19 and F20 showed RTAs above 35%.

Antithyroidal effects were not observed. None of the tested extracts was able to produce any decline in the hormone induction and the response obtained from the co-incubation (feed extract plus hormone) corresponded to the effect provoked by the hormone alone, indicating the absence of interferences.

4. Discussion

In the present study, potential endocrine disruption by fish diets was assessed by means of *in vitro* bioassays. Estrogenic activity was evaluated by using the newly developed HER-LUC reporter gene assay, whose development, optimization and validation is described in the present work. This reporter assay allowed the quantification of sbERα-mediated luciferase activity by hormones (E2, 17-α-estradiol), analogs or environmental samples. The bioassay is based on the HEK-293 cell line, stably expressing the sbERα and the luciferase under the control of EREs. The use of sbERα is of particular interest and was chosen taking into account that sea bass is a carnivorous species of great importance for Mediterranean aquaculture. Disruption at thyroidal level was screened with an already established reporter gene assay which expresses luciferase gene under the control of TRα1 of avian origin (Jugan et al., 2007). Our study is the first to demonstrate thyrogenic activity in fish diets commercially available and widely used in aquaculture.

Previous reports on endocrine disruption of feed or environmental samples use the responses obtained with different concentrations of the hormone standard as a reference to determine the endocrine activity of the studied sample (Matsumoto et al., 2004). In this study, dose–response curves obtained with the extracts of T3 or E2-spiked feed were used as reference. This approach allows a more accurate estimation of the activity observed in the feed samples, avoiding the under and over-estimation of activities, since extraction and treatment of the original hormone and of the sample are influenced by the same factors. Validation of the HER-LUC system demonstrated the previously described estrogenic effect of phenol red (Berthois et al., 1986). In addition, serum deprivation during treatment resulted in weaker reporter gene induction when cells were exposed to E2, again as previously reported (Ackermann et al., 2002). Moreover, the lack of serum appears to affect cell fitness since they are not able to overcome the high concentrations of fish feed extracts, resulting in cytotoxicity. Although the amplitude of the response to E2 diminished, the potency (EC_{50}) was in the same order of magnitude when using complete, phenol red-free, and charcoal treated serum/phenol red-free media. In light of these results and to reduce the phenol red and serum effects, all the assays were performed in phenol red-free medium supplemented with charcoal-treated serum. In these conditions, the EC_{50} value for E2 was 32 nM. Similar EC_{50} values were reported in previous reporter gene assays using fish ERs (Ackermann et al., 2002; Cosnefroy et al., 2009; Matthews et al., 2000). In the present work, the maximal induction factor obtained with HER-LUC cells was 54 fold. Previous studies have demonstrated that divergence in the amino acid sequences of the ligand binding domain of ER (Pakdel et al., 2000) resulted in lower affinity of rainbow trout ER (rtER) compared with human ER (hER) (Le Drèan et al., 1995). Indeed, the clearly discernible differences in sensitivity of the HER-LUC cells to estrogenic compounds could also be related with a lower binding affinity of sbERα for estrogens and estrogen-like substances than observed in rtER or hER. More experiments focusing on sbER binding affinity are required to corroborate this hypothesis.

To verify the correct functioning of the bioassay, the response to three agonists exhibiting different binding affinities for ER was assessed. Moreover, the effects of tamoxifen on E2-induced luciferase activity were also evaluated (Brzozowski et al., 1997; Navas and Segner, 2001). The detection of estrogenic activity in E2 and 17-α-estradiol demonstrated the specificity of the cell line to detect estrogenic substances with diverse efficacy. The RAA of 17α-estradiol was 100 times lower compared with E2 induction, which is in good agreement with the results obtained by Sonneveld et al. (2006). The representative hormone

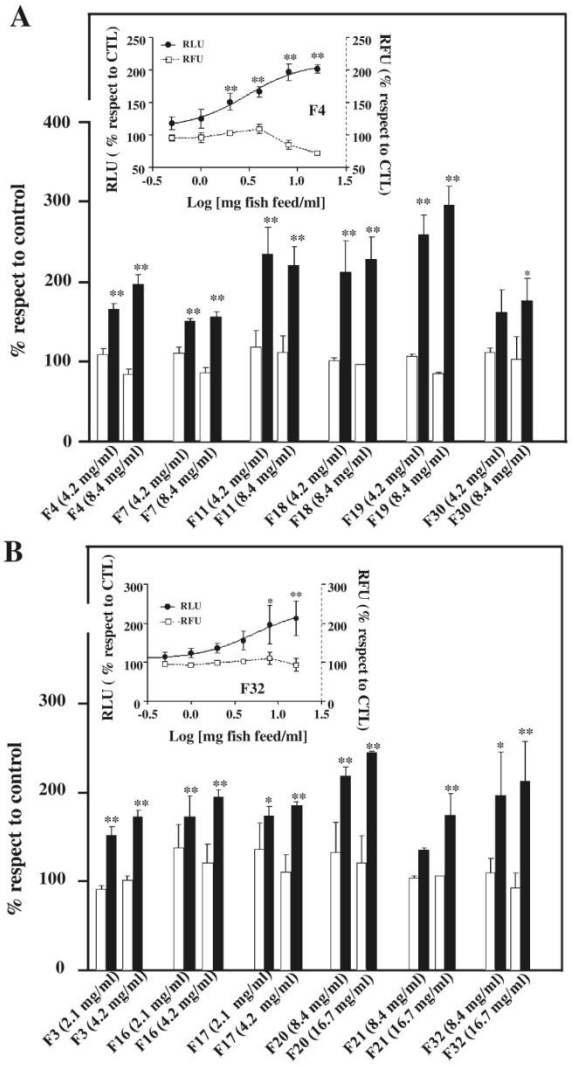

Fig. 6. Thyroidal activity represented as relative luminescence units (RLU, black) and cytotoxicity as relative fluorescence units (RFU, white) in PC-DR-LUC cells. The figure shows the induction of luciferase activity in 12 representative feeds at the two consecutive concentrations provoking the maximum effect. Columns represent the mean of at least two independent experiments ± SEM. The insets show the full dose response curves belonging to two of the samples F4 (6A) and F32 (6B). In the case of F4, the highest concentration (16.7 mg/ml) resulted cytotoxic to the cells, and although shown in the graph, was excluded from the RTA and EC_{50} calculations. Asterisks show statistical significant differences with respect to corresponding (luminescence or fluorescence) controls (*: $p<0.05$; **: $p<0.01$).

for the androgen receptor (17-α-methyltestosterone) showed a low agonistic response ($RAA = 8.53E^{-5}$). In addition, cells transfected only with ERE-TK-LUC construct were not responsive to E2 treatment, confirming that the luminescence response is only due to the activation of the ER. The inhibition of the estrogenic response due to pre-treatment with tamoxifen in a dose-dependent manner demonstrates that the induction of luciferase activity by E2 is specifically mediated by sbERα. However, we did not observe the partial agonistic action of tamoxifen at the low concentrations previously reported by other authors using mammalian cells or yeast expressing hER (Andersen et al., 1999; Legler et al., 1999). Similar results were obtained in previous experiments using rtER, suggesting species-specific differences in the ER response to tamoxifen. To further characterize the bioassay, the ability to detect estrogenicity in environmental samples was tested. Cells were exposed to extracts of effluents from wastewater treatment plants. The response obtained was similar in amplitude to that of E2, allowing calculation of the estrogenic potential and demonstrating the potential use of this cellular system for the study of environmental estrogenicity.

The estrogenicity caused by commercial fish feeds has been reported in previous studies (Beresford et al., 2011; Matsumoto et al., 2004; Miyahara et al., 2003). In the present work, 11 out of 32 diets induced sbERα-mediated transcriptional activity. The viability of the cells after exposure to feed extraction was assessed simultaneously to the reporter assays by means of the resazurin method. The decrease in fluorescence can be attributed to cell death and was useful for discarding cytotoxic concentrations in both cell lines. Interestingly, three feeds (F3, F9 and F31) in HER-LUC cells resulted in an increased fluorescence at specific concentrations. The increase in fluorescence has been previously used to estimate cell proliferation (Freitas et al., 2011). However, in our case, this increase was not due to cell proliferation, which was assessed by means trypan blue stain (data not shown). These results were probably related with an increase in the general activity of the cells in an attempt to metabolize the feed extract. The estrogenic capacity of these diets in the HER-LUC cells was weak, as shown by the RTA values. However, estrogenic diets have been shown to severely impact fish reproductive physiology (Pelissero and Sumpter, 1992). Our previous studies demonstrated that estradiol-supplemented diets have profound negative effects on food intake levels and growth performance in sea bass, suggesting that estrogenic diets could induce similar effects (Leal et al., 2009). Although RTA levels in the diets seemed low, it should be taken into account that absolute doses delivered daily to animals via food intake could reach 10^3–10^5 times those reported in the assays depending on the fish size and food intake level.

To our knowledge, this work is the first to report the detection of thyroidal activity of fish feed extracts. The present study shows that 56% (18 out of the 32) of the methanol extracts from fish diets tested have thyromimetic effects. Thyroid activity of fish diets was screened with the PC-DR-LUC reporter gene assay expressing luciferase under the control of TRα1 of avian origin (Jugan et al., 2007). The differences in receptor binding affinities or receptor–DNA interactions should be considered. For instance, rainbow trout nuclear TR showed a lower affinity for T3 than other animal species receptors (Ichikawa et al., 1989). When receptor–DNA interactions were compared among species, the receptors from rainbow trout and dog liver were similar. In spite of these differences, the use of an already established assay, as a first approach in assessing potential thyroid disruption in fish feeds, was considered the correct option. Future approximations should include the development of a cell line transfected with a TR from a fish species (e.g. rainbow trout or sea bass), not described to date.

A number of studies have reported the disruption of thyroid homeostasis by numerous industrial chemicals, such as polychlorinated biphenyls (PCBs), dioxins, flame retardants, including polybrominated diphenyl ether (PBDEs), phenolic compound and their halogenated derivatives, phthalates, and pesticides (Boas et al., 2006). The presence of thyroid endocrine disruptors in wastewater treatment plants, river and drinking water (Jugan et al., 2009) has also been evaluated, most activity being related with hydrophobic compounds. However, in our case the extracts performed with hexane did not provoke any induction of the TR-mediated luciferase activity, or the inhibition of the activation caused by treatment with T3. Thus, we might speculate that the thyroidal activity found in the fish feeds is mainly due to polar compounds, including hormones and some pollutants, but not to industrial chemicals exhibiting low polarity. However, this assumption must be confirmed through more specific analyses.

Thyroid hormones are essential in metamorphic transformation processes and play a crucial role in the postnatal maturation of different organs during early development (Blanton and Specker, 2007; Carr and Patiño, 2011; Flamant and Samarut, 2003). Experiments have demonstrated that deiodinase activity regulates pigmentation

in zebrafish (Walpita et al., 2009) and treating flatfish with T4 increases the rate of albinism (Yoo et al., 2000). Positive correlation has been found between thyroid status and reproductive status (Cyr and Eales, 1996). In zebrafish the exogenous administration of TH produced strongly male-biased cohorts, whereas the testis weight and gonadosomatic index was 100% higher in tilapia treated early with goitrogens than in control fish. Treated tilapia also exhibited retarded growth (Matta et al., 2002). The presence of thyromimetic compounds in fish diets could severely compromise early developmental processes, resulting in unwanted characteristics in reared fish.

In conclusion, this report describes for the first time the simultaneous assessment of estrogenic and thyroidal activities in commercial fish feed. Although the estrogenic activity was weak, in more than half of the diets a very high thyroidal activity was detected. In addition, five of the fish feeds, produced simultaneously estrogenic and thyromimetic activity *in vitro*. Moreover, given that fish receive food continuously, the observed effects could be multiplied with time and with the quantity of ingested food, so that the estrogenic or thyroidal activity observed should be taken into account with regards to its potential impact on fish population in aquaculture.

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CHAPTER III:
Thyroid signaling in the piscine immune system.

PAPER IV:

THYROID SIGNALING IN IMMUNE ORGANS AND CELLS OF THE TELEOST FISH RAINBOW TROUT (*ONCORHYNCHUS MYKISS)*

RESUMEN

En los mamíferos, se sabe que las hormonas tiroideas modulan el sistema inmune. Sin embargo, se desconoce el rol que juegan esas hormonas en el sistema inmune de los peces teleósteos. En este estudio se presenta evidencia de la existencia de una señalización tiroidea activa en órganos y celulas inmunes de los peces teleósteos. Demostramos que los principales órganos inmunes (riñón cefálico y bazo) así como leucocitos aislados (del riñón cefálico y sangre) de la trucha arcoiris (Oncorhynchus mykiss) expresan los receptores de hormonas tiroideas α (THRA) y β (THRB). Los valores de cuantificación absoluta de mRNA de THRA fueron significativamente mayores que aquellos de THRB. THRA mostró una mayor expresión génica en los órganos inmunes y en las células aisladas en comparación con el órgano de referencia, el hígado mientras que THRB mostró lo contrario. La exposición *in vivo* de truchas a triiodotironina (T3) o a el agente anti-tiroideo propylthiouracil (PTU) alteró la expresión génica de los receptores en los órganos inmunes así como en las células. El efecto de la T3 y PTU sobre la expressión de marcadores génicos de subpoblaciones de células inmunes también fue estudiado. Los tratamientos alteraron la expresión génica de marcadores de linfocitos T (cd4, cd8a y trb), linfocitos B (mIgM) y macrófagos (csf1r). Estos resultados sugieren que el sistema inmune de la trucha arcoiris responde a las hormonas tiroideas.

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Full length article

Thyroid signaling in immune organs and cells of the teleost fish rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

Thyroid hormones are involved in modulating the immune system in mammals. In contrast, there is no information on the role played by these hormones in the immune system of teleost fish. Here we provide initial evidence for the presence of active thyroid signaling in immune organs and cells of teleosts. We demonstrate that immune organs (head kidney and spleen) and isolated leukocytes (from head kidney and peripheral blood) of the rainbow trout (*Oncorhynchus mykiss*) express both thyroid receptor α (THRA) and β (THRB). Absolute mRNA levels of THRA were significantly higher than those of THRB. THRA showed higher expression in immune organs and isolated immune cells compared to the reference organ, liver, while THRB showed the opposite. *In vivo* exposure of trout to triiodothryronine (T3) or the anti-thyroid agent propylthiouracil (PTU) altered THR expression in immune organs and cells. Effect of T3 and PTU over the relative expression of selected marker genes of immune cell subpopulations was also studied. Treatments changed the relative expression of markers of cytotoxic, helper and total T cells (*cd4*, *cd8a*, *trb*), B lymphocytes (*mIgM*) and macrophages (*csf1r*). These findings suggest that the immune system of rainbow trout is responsive to thyroid hormones.

1. Introduction

Thyroid hormones (THs) play critical roles in growth, metabolism, and development in all vertebrates, including fish (reviewed in Ref. [53]). TH production is regulated by the hypothalamus–pituitary–thyroid (HPT) axis through positive and negative feedback mechanisms. In mammals, thyrotropin-releasing hormone (TRH) produced in the hypothalamus, stimulates the pituitary to release thyroid-stimulating hormone (TSH). However, in teleost fish this fact is less well established, and instead, corticotrophin-releasing hormone (CRH) appears to play an important role as a TSH-releasing factor (reviewed in Ref. [18]). TSH in turn, controls the secretion of L-thyroxine (T4) by thyroid follicles. Once in circulation, T4 enters target cells, where it undergoes monodeiodination, thus being converted into the biologically active 3,3′,5-triiodo-L-thyronine (T3). Correct function of the HPT axis is essential for the proper development and reproduction of animals (reviewed in Refs. [4,7]). In fish and amphibians, THs are responsible for the changes that occur during metamorphosis. For instance, the transformation of flounder larvae into juveniles has been shown to be dependent upon THs [52,53] and exposure to TH induces early metamorphosis in zebrafish [10].

In vertebrates, the action of THs is multifaceted and goes beyond development and reproduction [45,50,68]. One target of TH action appears to be the immune system. In mammals, there is a bi-directional and complex relationship between the HPT axis and the immune system (reviewed in Refs. [19,33]). For instance, the cells of the immune system produce TSH [64] and the presence of T3 has been reported in white blood cells and mast cells [16,48]. At the same time, thyroid receptors are present in mammalian immune cells, and THs have been found to influence the distribution of lymphocyte subsets [44,47] and to modulate specific immune responses, including cell-mediated immunity [9], B lymphocyte differentiations [46], natural killer cell activity, and T lymphocyte proliferation [27,54]. In addition, immune functions such as phagocytosis, the generation of reactive oxygen species (ROS), and the synthesis and release of cytokines are affected by hypo- and hyper-thyroid conditions (reviewed in Ref. [19]).

In teleost fish, THs have been studied mainly with regard to their function in development, metamorphosis, growth, and

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reproduction (i.e.: [3,14,15,37]). In contrast, there is scarce information about a possible immunomodulatory role of THs in fish [70], although a few studies have provided indirect evidence that THs influence piscine immune parameters. Slicher [63] reported significantly reduced numbers of circulating leukocytes in hypothyroid fish. Correspondingly, Ball and Hawkins [1] observed that the administration of thyroxin or TSH recovered the number of circulating lymphocytes in hypo-physectomized killifish. More recently, an immunostimulating role of T3 has been suggested, based on the increased survival of T3-fed rohu (*Labeo rohita*) to *Aeromonas hydrophila* [59]. The involvement of THs in thymus development is supported by a study performed by Lam et al. [35]; who found that T4 increased thymus size in developing zebrafish, while the anti-thyroid agent methimazole caused the opposite effect. Information on a possible immunomodulatory role of THs in teleost fish is relevant with respect to comparative endocrinology and immunology. But, in addition, such information is significant with regard to potential impacts of thyroid-disrupting chemicals present in the environment (reviewed in Refs. [8,32,34]) to which natural fish populations and aquaculture facilities may be exposed [55].

THs exert their actions upon target organs and cells through binding to their receptors (THRs), which are ligand-dependent transcription factors that interact with specific DNA regions to regulate the expression of a wide range of genes. To date, two main subtypes of THRs, THRA and THRB have been identified in vertebrates. In fish, they are the product of at least two (i.e.: rainbow trout; [29,39] or three (i.e.: Japanese flounder; [71,72] distinct genes which can, in turn, generate several isoforms by alternate splicing.

In mammals, THRA and THRB are expressed in the organs and cells of the immune system (e.g.: Refs. [5,26,42]), and they mediate a number of immune effects exerted by THs [6,49]. Here we sought to unravel whether THRs are also expressed in immune organs and cells of teleosts and to evidence the effects of TH status on immune function. In this regard, we examined the following: (i) THRA and THRB expression in immune organs and cells of rainbow trout (*Oncorhynchus mykiss*); (ii) the levels and ratios of the two receptor subtypes expressed in these tissues and how they compare with the liver as a reference organ; (iii) alterations in immune THR expression in response to treatment with THs or anti-thyroid agents; and (iv) the association between these changes and altered ratios of marker genes of immune cell subpopulations.

2. Materials and methods

2.1. Fish samplings and T3 and PTU exposure experiment

Juvenile rainbow trout (*O. mykiss*, 49 ± 2.7 g body weight) were selected from the stock population reared at the Centre for Fish and Wildlife Health, University of Bern, Switzerland.

The fish used for exposure experiment were distributed into six glass aquaria, each with a volume of 200 L (ten fish per aquarium). They were kept under flow-through conditions (0.5 L/min) in air-saturated tap water (total hardness ($CaCO_3$): 2.13 ppm; chlorine dioxide: <0.01 mg/l) and at a water temperature of 15 ± 1 °C under a natural light–dark photoperiod.

Two groups (each with two replicates of 10 fish) were fed daily (1% weight) with a commercial diet spiked either with 20 μg/g of T3, or with 5000 μg/g of the anti-thyroid agent 6-Propyl-2-thiouracil (PTU). T3 and PTU were dissolved in ethanol and sprayed over the pellets, which were then dried at 60 °C to allow solvent evaporation. The control group (two replicates, each with 10 fish) was fed pellets exposed to the same percentage of ethanol. Test concentrations and duration of the exposure were selected following information available in the literature (For T3: Finsson and Eales [22] working on *O. mykiss*; Takagi et al. [66]; working on *O. mykiss*. For PTU: Peter and Peter [51]; working on tilapia (*Oreochromis mossambicus*); Sullivan et al. [65]; working on chum (*Oncorhynchus keta*), coho (*Oncorhynchus kisutch*), chinook (*Oncorhynchus tschawytscha*), and Atlantic salmon (*Salmo salar*). In those studies, the selected doses led to changes in plasma hormones (T3/T4) as well as in deiodinase activity and gene expression.

At the start of the experiments (day 0) nine animals were sampled for determining THR basal expression. Samplings of 10 fish per group (i.e. five fish per replicate) were performed at days 7 and 15 after the start of exposure, and blood and tissues of each two animals were pooled. Fish were immediately sacrificed with 100 mg/l of tricaine methane sulphonate (MS-222, Argent Chemical Laboratories, Redmont CA, USA). Animal fork length and mass were recorded and liver and gonads weighed. The hepatosomatic index (HI) was calculated following [24]. Immune cells from the head kidney and blood were isolated (see below), and RNA was immediately extracted. Samples from the liver, head kidney, and spleen were stored in RNA-later (Sigma–Aldrich, Buchs, Switzerland) until RNA extraction.

2.2. Leukocyte isolation

Immune cells from the head kidney and blood were isolated using the Ficoll-Hypaque method (Biochrom AG, Berlin, Germany). Briefly, 1 mL of blood (pool of 500 μL from two animals belonging to the same group) withdrawn from the caudal vein was diluted 1:10 with L-15 medium (Gibco, Basel, Switzerland) containing 10 U/mL of heparin. In the case of the head kidney, approximately 0.5 mg of tissue (pool of two animals belonging to the same group) was homogenized by repeated pipetting with 10 mL of L-15, and the resulting homogenate was passed through 250-μm and 125-μm nylon meshes. Ten mL of the resulting cell suspension (either blood/L-15 or head kidney/L-15) was then placed onto 3 mL of Ficoll (1.077 g/ml; Biochrom AG, Berlin, Germany) and centrifuged for 40 min at 400 × *g* at 4 °C. The layer containing the leukocytes was transferred to a fresh tube and washed several times by centrifugation (10 min at 400 × *g* 4 °C). The final pellet was lysed in TRI® reagent and maintained at −80 °C until RNA extraction.

2.3. RNA extraction and synthesis of first strand cDNA

Total RNA was extracted from tissues and isolated immune cells using TRI® reagent, following the manufacturer's instructions (Sigma–Aldrich, Buchs, Switzerland). After DNA digestion with RQ1 RNase-Free DNase (Promega AG, Wallisellen, Switzerland), RNA concentration and purity was measured with NanoDrop ND-1000 (NanoDrop Technologies Inc., Wilmington DE, USA). Subsequently, reverse transcription (RT) was carried out in 20 μL of reaction volume containing 500 ng Random Primers, 2.5 mM $MgCl_2$, 2 mM dNTPs, 20 U RNAse inhibitor, 160 U M-MLV reverse transcriptase (all Promega AG, Wallisellen, Switzerland) and 500 ng total RNA. cDNA was kept at −20 °C until quantitative PCR (qPCR) was performed.

2.4. PCR primers

Primers for THRs (*thra* and *thrb*) and deiodinase type 2 (*dio2*) were designed using sequences obtained from GeneBank (NCBI). As immune markers, T cell receptor, beta chain (*trb*), *cd4* and *cd8a* were selected to monitor changes in T cell dynamics [56]. T-cell receptor is expressed on the surface of all T cells, while CD8α and CD4 distinguish between subpopulations of T cells (cytotoxic T cells and helper T cells, respectively). Macrophage Colony-Stimulating

Factor-1 Receptor (*csf1r*) was used as macrophage marker gene [58,69]. Changes in B-cell population were inferred from the expression of membrane IgM (*mIgM*) [74]. Primers for the lymphocyte receptors *trb*, *cd4*, *cd8a* and *mIgM* were selected from the indicated references, all corresponding to rainbow trout while those for the *csf1r* were designed using rainbow trout sequences obtained from GeneBank (NCBI). Given the constant expression of elongation factor 1-alpha (*eef1a*) among tissues and treatment groups—which was checked in a preliminary study (data not shown)—it was used as housekeeping gene [23] (Table 1).

2.5. RT-qPCR

We performed qPCR measurements in an Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using the following protocol: pre-incubation steps at 50 °C for 2 min and 10 min at 95 °C, followed by 40 amplification cycles (10 s at 95 °C and 50 s at 60 °C). In order to ensure the amplification of the correct product, a dissociation curve analysis (heating from 56 °C to 95 °C) was also performed. PCR products were sequenced to verify that the reaction amplified the proper sequence.

Each reaction mix contained 1X FastStart Universal SYBR Green Master (Roche-Applied Biosystems, Rotkreuz, Switzerland), 300 nM of each primer, and 1 μL of cDNA. Relative quantification was achieved using the comparative Ct method, also referred to as $2^{-\Delta\Delta\ Ct}$, representing the amount of target gene normalized to the endogenous control gene (*eef1a*) and relative to the mean value of the control group. That is, $\Delta\Delta Ct = (Ct\ target - Ct\ eef1a)_{treated\ fish} - (Ct\ target - Ct\ eef1a)_{control\ group.}$

In the case of *thrs*, absolute quantification was performed. Absolute quantification is widely used nowadays (e.g. Refs. [11,21]). The method applied in the present study performed quantification based on standard curves generated from purified RT-PCR products. More specifically, we produced a standard solution for each primer with head kidney cDNA as described in Refs. [61,17].

2.6. Statistical analysis

The results are expressed as mean ± standard error of the mean (sem). All the statistical analyses were performed with Sigma Plot 12.0. Normality of data was checked by Kolmogorov–Smirnov test, while homogeneity of the variances was automatically checked by Sigma Plot 12.0 by observing variability between group means. Differences in *thrs* expression between organs were determined with one-way analysis of variance (ANOVA) followed by Tukey's test. Significant differences were accepted at $p < 0.05$. Differences in gene expression between each of the treatments (T3 or PTU) and control were determined with ANOVA, followed by Dunnet's test. Significant differences were accepted at $p < 0.05$.

3. Results

3.1. Expression of thra and thrb in immune organs and cells of control rainbow trout

To explore whether the immune organs and total immune cells of the rainbow trout express *thrs*, we used RT-qPCR to measure mRNA levels of *thra* and *thrb* in spleen, total head kidney (which, in addition to immune cells, comprises other cell types such as vascular endothelial cells, and endocrine cells), and pure immune cell populations isolated from the head kidney and blood. In parallel, *thra* and *thrb* levels were measured in the liver as reference organ.

Both receptor subtypes were ubiquitous, although absolute expression varied among tissues (Fig. 1). *Thra* showed higher expression in the immune organs and cells isolated from the blood and head kidney than in the liver. *Thra* copy numbers in immune organs and isolated cells ranged between 1750 ± 435 (isolated cells from the blood) and 2812 ± 278 (head kidney), while liver presented only 239 ± 35 copies. In contrast, *thrb* expression was higher in the liver (960 ± 210 copy numbers) than in immune cells and organs. *Thrb* levels ranged from 130 ± 72 (cells isolated from the head kidney) to 701 ± 132 copy numbers (spleen). Regarding the differences between whole organ (head kidney) and cells isolated from the head kidney, both *thrs* exhibited higher expression in the organ, indicating that they are also expressed in non-immune cells. However, we found a clear difference between *thra* and *thrb* expression. While *thra* showed a similar level in the organ (2812 ± 278 copies) and isolated cells (2534 ± 584 copies), *thrb* did not (621 ± 81 vs. 129 ± 72 copies respectively). These observations thus indicate that *thra* is expressed in immune cells while *thrb* is found mainly in non-immune cells.

Differences in *thr* expression between immune cells isolated from the blood and those isolated from the head kidney were not

Table 1
Primers for quantitative PCR analysis of gene expression.

Target gene (Accession number)	White cell detected	Primer sequence (5′–3′)	Product size (bp)	Reference
eef1a (NM_001124339.1)	–	S: TGCCCCTGGACACAGAGATT AS: CCCACACCACCAGCAACAA	90	[23]
thra (AF302245)	–	S: TGGGAGCTGATCCGCATG AS: CCTTGTCTCCGTCCGAC	136	This study
thrb (AF302246)	–	S: TGGGAGCTGATCCGCATG AS:GCTCCCTTCAGGTGCATTTATG	160	This study
dio2 (AF312396.1)	–	S: ATCACTGGAAGAAAGGGTGG AS: TCTCGTTGGACACACCGTAG	128	This study
cd8a (AF178054)	Cytotoxic T cells (Tc)	S:ACACCAATGACCACAACCATAGAG AS: GGGTCCACCTTTCCCACTTT	74	[56]
cd4 (AY973028)	Helper T cells (Th)	S: CATTAGCCTGGGTGGTCAAT AS: CCCTTTCTTTGACAGGGAGA	89	[56]
trb (AF329700)	Total T cells	S: TCACCAGCAGACTGAGAGTCC AS:AAGCTGACAATGCAGGTGAATC	73	[56]
mIgM (AH014877.2)	B lymphocytes	S: CCTTAACCAGCCGAAAGGG AS: CCAACGCCATACAGCAGAG	93	[74]
csf1r (NM_001124738)	Macrophages	S: CAGAGCGTCCTGACAATGGA AS: GGCGAAGGTGTCTTTACCGA	74	This study

S: sense.
AS: antisense.

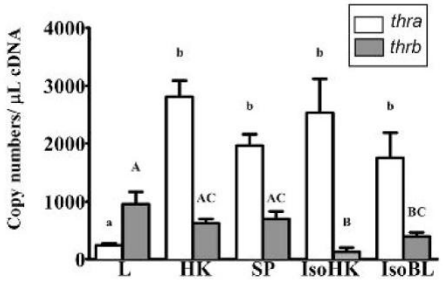

Fig. 1. Basal absolute expression of *thra* (white bars) and *thrb* (gray bars) in several organs of juvenile rainbow trout: liver (L), head kidney (HK), spleen (SP), immune cells isolated from head kidney (isoHK), and immune cells isolated from blood (isoBL), as determined by RT-qPCR. Values are mean ± sem ($n = 9$). Significant differences between organs are indicated by lower (*thra*) or upper (*thrb*) case letters ($p < 0.05$).

significant. The former showed a slightly lower *thra* expression (1750 ± 435 copies) compared to the latter (2534 ± 584 copies), while mature blood cells showed greater *thrb* expression (388 ± 70 copies) than immune cells of the head kidney (130 ± 72 copies).

3.2. Experimental treatment of rainbow trout with T3 or PTU

3.2.1. Effects of T3/PTU on length, weight, condition factor and HI

Juvenile rainbow trout were exposed through the diet to either T3 (20 µg/g) or PTU (5000 µg/g). Treatments did not lead to significant changes in body length, body weight or condition factor, except for the group that received T3 for 15 days, which showed an increase in both body weight and length, although condition factor was not altered. HIs were lower in the T3 and PTU groups than in controls after 7 and 15 days of treatment (Table 2). No fish died in any of the groups during the experiment.

3.2.2. Effects of T3/PTU on dio2, thra and thrb mRNA levels in liver

The liver was analyzed as reference organ. Exposure of trout to T3 did not significantly change hepatic transcript levels of *thra* compared to control fish (Fig. 2A). In contrast, PTU treatment resulted in a decrease in this parameter at day 15 (Fig. 2A). T3 administration caused an increase in transcript levels of *thrb* after 15 days, whereas the PTU treatment had no effect (Fig. 2B).

In addition to the THR levels, we analyzed hepatic transcript levels of *dio2*. Treatment with T3 (Fig. 2C) led to a decrease in the relative abundance of *dio2* transcripts at both sampling dates, with final levels being approximately 25% of those detected in controls. Conversely, fish exposed to the anti-thyroid agent PTU showed an increase in *dio2* expression at day 7 of treatment, with *dio2* transcripts increasing approximately 2.5–3-fold compared to control fish. Similar values were observed at day 15 of treatment, although they were not significantly different from controls.

3.2.3. Effects of T3/PTU on thra and thrb mRNAs in immune organs and isolated immune cells

Fish fed T3 displayed significantly elevated transcript levels of *thra* and *thrb* in the spleen after 7 days (Fig. 3A and B). At day 15,

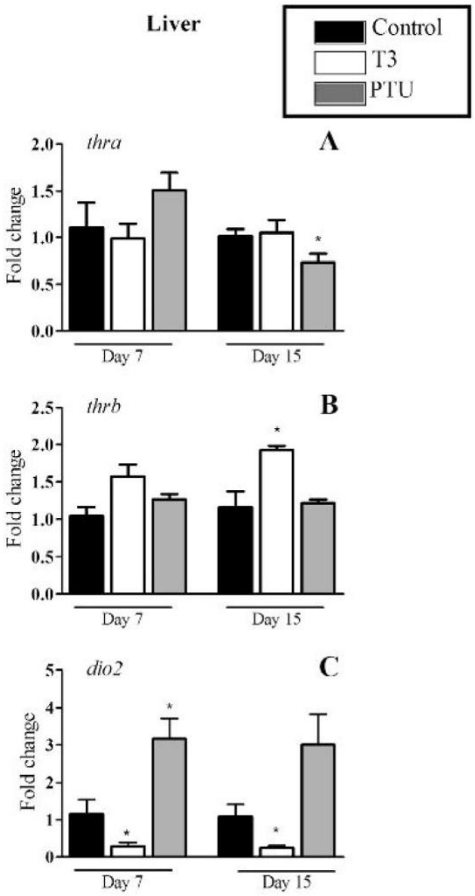

Fig. 2. Relative expression of *thra* (A), *thrb* (B) and *dio2* (C) in liver of juvenile rainbow trout after 7 and 15 days of exposure to T3 (white) and PTU (gray). Control group is shown in black. Values are mean ± sem ($n = 5$). Significant differences (*) between treatment and control groups were considered at the $p < 0.05$.

they had returned to control levels. PTU treatment had no effect on splenic *thra* or *thrb* mRNA levels.

In the head kidney, relative transcript abundance of *thra* and *thrb* was not altered by PTU or T3 treatment (data not shown).

Leukocytes isolated from the head kidney showed a decrease in the relative transcript abundance of *thra*, both after 7 and 15 days of T3 treatment (Fig. 4A). A similar trend was observed for *thrb* mRNA levels, although the decrease was not significant (Fig. 4B). For the PTU treatment, effects were observed only at day 15, with both *thra* and *thrb* mRNA levels being almost completely repressed ($p < 0.05$). In immune cells isolated from blood (Fig. 4C and D) T3 treatment caused a decrease in *thra* and *thrb* mRNA levels after 7 days of treatment, leading to almost complete repression of both genes, but after 15 days of treatment mRNA levels for these two receptors were similar to those of controls. For the PTU treatment, *thra* and *thrb* mRNA levels increased at day 7. In contrast, after 15 days of treatment those of *thrb* decreased (Fig. 4C and D).

Table 2
Body weight, body length, condition factor and hepatosomatic indexes in the *in vivo* experiment.

Parameter	Control day 7	T3-group Day 7	PTU-group Day 7	Control day 15	T3-group day 15	PTU-group day 15
Weight (g)	53.00 ± 2.38	56.70 ± 3.33	48.70 ± 2.66	52.50 ± 3.00	61.11 ± 2.74*	56.66 ± 2.36
Length (cm)	17.00 ± 0.35	17.60 ± 0.32	16.76 ± 0.27	16.89 ± 0.27	18.11 ± 0.22*	17.83 ± 0.30
Condition factor	1.08 ± 0.33	1.03 ± 0.03	1.03 ± 0.02	1.08 ± 0.03	1.02 ± 0.02	1.03 ± 0.04
Hepatosomatic index	1.65 ± 0.09	1.36 ± 0.09*	1.26 ± 0.08*	1.54 ± 0.06	1.33 ± 0.06*	1.29 ± 0.10*

Data represent mean ± sem ($n = 10$). Significance: *: $p < 0.05$.

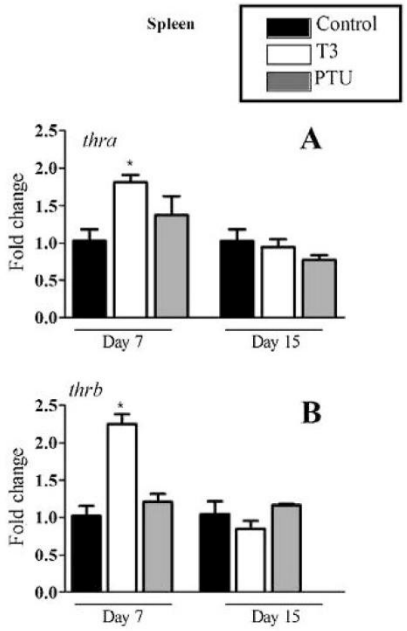

Fig. 3. Relative expression of *thra* (A) and *thrb* (B) in spleen of juvenile rainbow trout after 7 and 15 days of exposure to T3 (white) and PTU (gray). Control group is shown in black. Values are mean ± sem ($n = 5$). Significant differences (*) between treatment and control groups were considered at the $p < 0.05$.

3.2.4. Effects of T3/PTU on transcript levels of leukocyte markers in immune cell populations isolated from the head kidney and blood

The gene expression of the five molecular markers (*trb*, *cd4*, *cd8a*, *csf1r* and *mIgM*) corresponding to different subpopulations of white blood cells was analyzed by RT-qPCR in immune cells from the head kidney and blood.

In cells from the head kidney (Fig. 5, A–E), T3 treatment led to a decrease in the levels of T-lymphocyte markers (*trb*, *cd4* and *cd8α*) and in *mIgM* at day 7. This effect disappeared after 15 days of treatment. However, treatment with T3 had the opposite effect on *csf1r*, which showed an increase at day 7 but a decrease after 15 days of exposure (Fig. 5E). The effects of PTU on the expression of cell subpopulation markers were similar to those caused by T3, with a decrease in *trb*, *cd4*, *cd8a* and *mIgM*, and an increase in *csf1r* at day 7. Like T3, PTU also produced a decrease in *csf1r* mRNA levels after 15 days of treatment.

Regarding blood leukocytes (Fig. 5, F–J), T3 had no significant effect on any of the immune cell markers after 7 days of treatment. After 15 days of exposure, T3 caused a decrease in *cd4* and in *mIgM* mRNA levels and an increase (not statistically significant) in *cd8α*. Treatment with PTU over 7 days caused effects in line with those found for T3, namely no effect on T cell markers but an increase in *mIgM* and *csf1r* expression. After 15 days, PTU produced an increase in *cd8a* and a decrease in *mIgM* and *csf1r* mRNA levels.

4. Discussion

Here we sought to unravel whether THRs are present in the immune organs and cells of rainbow trout and to obtain initial insight into whether these receptors are functional parameters. Our results show, for the first time in a teleost species, that purified leukocyte cells have high expression levels of THRs, that THR mRNA expression in immune cells is in the range or even higher than that found in the liver, and that the dominant THR subtype in immune cells is THRA, in contrast to the liver, where THRB predominates.

Given that immune organs are composed of a mixture of immune cells and non-immune cells (stromal cells, endothelial cells, etc.), *thr* expression was not only analyzed in the total organ, but also in pure immune cell preparations isolated from the head kidney and blood, in order to unravel whether *thr* expression is indeed present in immune cells. The finding that *thr* mRNA is indeed expressed in immune cells of head kidney and blood of rainbow trout suggests an immunomodulatory role of the thyroid system.

Expression of *thra* and *thrb* differed between immune organs and livers. While *thrb* was found to be expressed mainly in the liver, *thra* was preferentially expressed in immune-related organs (spleen, head kidney) and in immune leukocytes (from the head

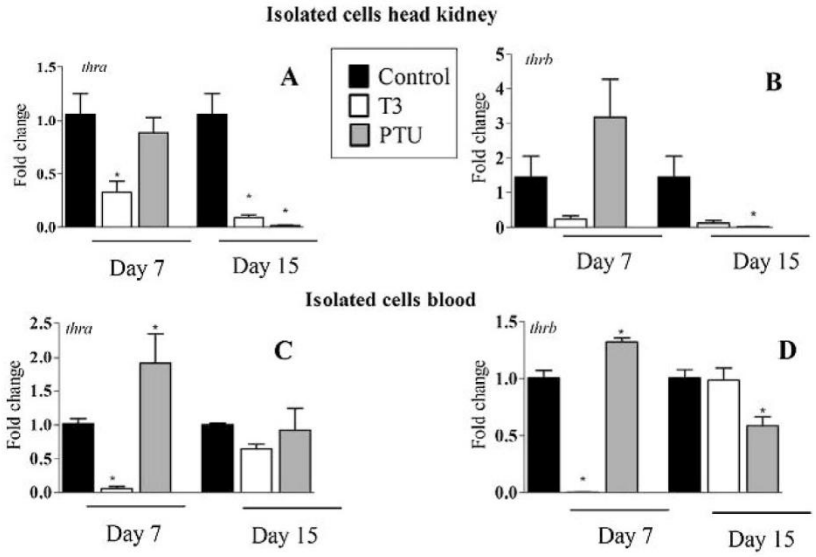

Fig. 4. Relative expression of *thra* and *thrb* in immune cells isolated from the head kidney (A, B respectively) and immune cells isolated from blood (C, D respectively) of juvenile rainbow trout after 7 and 15 days of exposure to T3 (white) and PTU (gray). Control group is shown in black. Values are mean ± sem ($n = 5$). Significant differences (*) between treatment and control groups were considered at the $p < 0.05$.

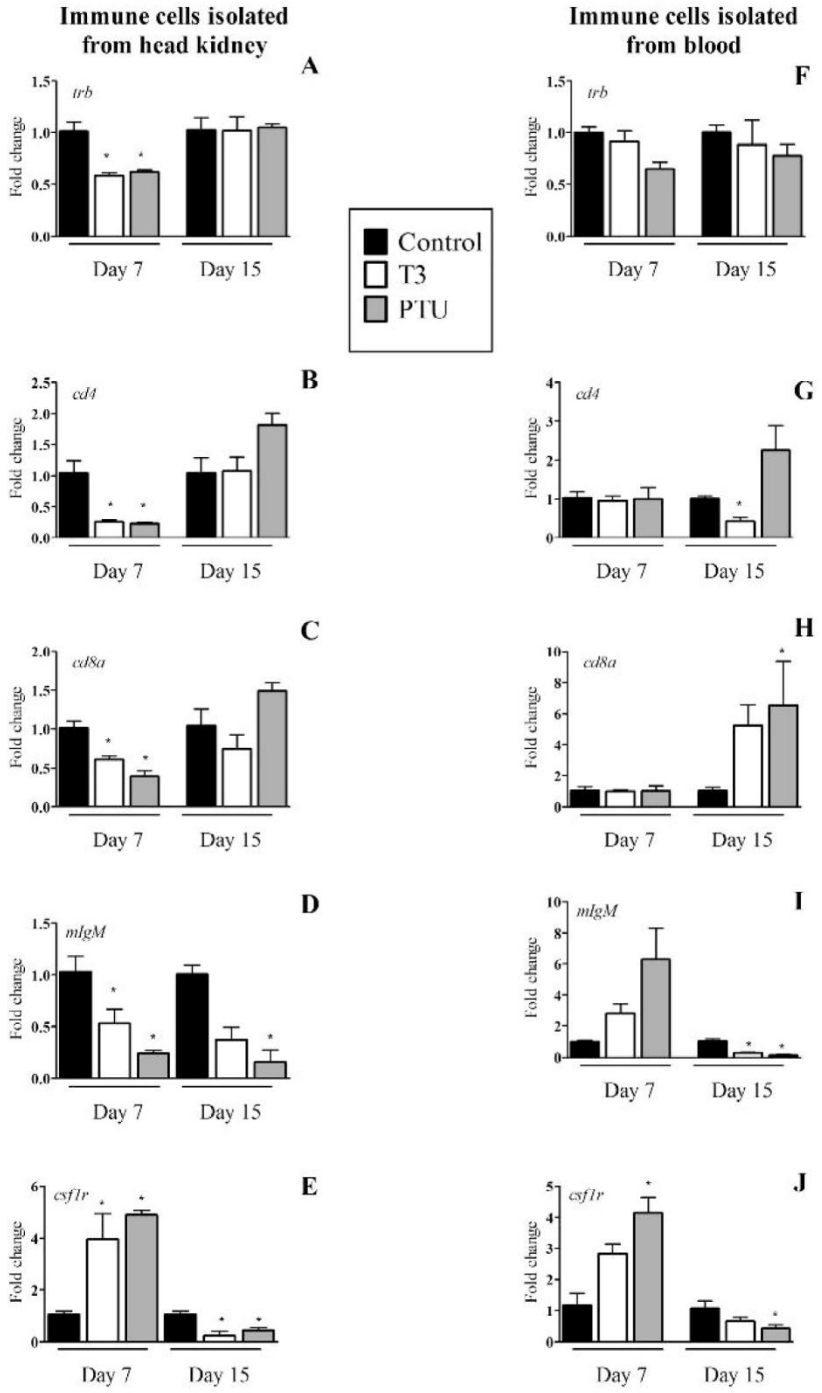

Fig. 5. Relative expression of *trb*, *cd4*, *cd8a*, *mIgM*, and *csf1r* in immune cells isolated from the head kidney (A–E) and blood (F–J) of juvenile rainbow trout after 7 and 15 days of exposure to T3 (white) and PTU (gray). Control group is shown in black. Values are mean ± sem ($n = 5$). Significant differences (*) between treatment and control groups were considered at the $p < 0.05$.

kidney and blood). A higher expression of *thrb* than of *thra* in liver has previously been reported in juvenile rainbow trout [57], rat [73] and humans [42]. The latter study also found higher expression of *thra* in human spleen and head kidney compared to liver [42]. In fish, many studies have assessed *thr* tissue distribution, but most of them have focused on organs such as liver, brain, pituitary and gonads [28,30,31,36,43]. Some studies have also found THR expression in immune organs such as thymus [67], spleen [12,40] and also in excretory kidney [25,62], which in fish contains hematopoietic parts, while the head kidney, which is a major immune organ in fish, appears to have not been studied yet. Also, given the fact that the immune organs are composed of a variety of cell types, from the previous studies, it is not clear whether the THRs are indeed expressed in the leukocytes.

Tissue-specific variation of *thra* and *thrb* expression has been commonly reported by several studies (i.e.: Refs. [25,31,38]) which is in agreement with our findings. This differential receptor subtype expression may imply that the subtypes serve different functions, probably in a tissue-specific manner [38]. In mammals, it has been suggested that THRB is involved in the regulation of pituitary, liver and cochlear function, whereas THRA regulates cardiac function, body temperature and lymphocyte development, in agreement with the respective predominant expression of these receptors across tissues [2]. In accordance with these reports in mammals, our results suggest that THRA is also the main mediator of the effects of THs on trout immune cell function.

Having demonstrated that both *thrb* and *thra* are present in the immune system of rainbow trout, we examined whether THR mRNA levels are responsive to the TH status of the organism. To this end, a 15-day *in vivo* experiment was carried out with juvenile rainbow trout. Treatment with exogenous T3 over 15 days caused a decrease in hepatic *dio2* gene expression while PTU exposure resulted in an overexpression of this gene. Since the main function of deiodinase 2 is the conversion of L-thyroxine (T4) to the more biologically active T3 [60], it has been used to monitor thyroid status changes in teleost species [28,41]. Our results indicate that the treatments administered affected the general thyroidal status of the animal, as has been demonstrated in other fish species (e.g., Ref. [28]). T3 or PTU treatment also affected the expression of the THR subtypes, with the effects being tissue-specific. While *thr* expression in liver and total immune organs showed little or no response to the treatments, the immune cells from head kidney and blood presented strong changes. Although it is too early to speculate about the functional implications of the specific sensitivity of the THRs in the immune cells to TH status, we wish to highlight two points. First, THRs in immune cells (7 days) responded much faster to treatments than THRs in the liver (15 days). This is another indication of a particular role of thyroid signaling in trout leukocytes. Second, the striking differences found between whole immune organ and isolated immune cells highlight the importance of using pure isolates of the target cell population when examining receptor expression and function in heterogeneous organs. While *thra* showed similar expression levels in the whole organ and the isolated leukocytes, *thrb* expression was clearly higher in the organ. This finding indicates that *thrb* is specifically expressed in the non-immune cell population of the head kidney.

A final question addressed in this study was whether changes of TH status affect the dynamics of leukocyte populations. Mammalian immune cell subpopulations show important differences in the expression of THRs, and this has consequences for thyroid-mediated alterations of immune cell differentiation and proliferation [20,26,46,49]. To explore whether changes in thyroid status affect leukocyte composition in rainbow trout, we measured several marker genes of macrophages, T lymphocytes and B lymphocytes. The effects of T3 and PTU treatments on leukocyte marker genes were tissue- and time-dependent. These findings are consistent with observations in the rat. For instance, PTU was found to alter helper (cd4+)/suppressor (cd8α+) T cell ratio, with increasing proportions in the spleen but decreasing indexes in the blood [47]. In another study, cd8α+ cells were found to decrease in hypothyroidism and increase in hyperthyroidism when measured in blood. However, in the spleen, differences were not significant [44]. Overall, the maintenance of the ratios of lymphocyte subpopulations in mammals appears to be strongly modulated by T3 levels [27], so that disruption of thyroid homeostasis could induce changes in the proportions of immune cells in mammals. Likewise, in the rainbow trout, we observed altered ratios of the marker genes after experimental manipulation of the TH status, what might be taken as a direct thyroid hormone effect on immune cell dynamics. However, care must be taken with such an interpretation. The fact that T3 and PTU treatments tended to induce similar effects could be indicative of indirect, not receptor-mediated effects taking place. With this study we know now that thyroid receptors are present in the teleostean immune system, but future work will have to unravel the processes and pathways through which thyroid signaling modulates immune functions of fish. Having observed in previous studies the presence of substances with thyroid-like effects in fish feed [55], we consider it pertinent to clarify whether thyroid disruption-mediated immunomodulation entails an environmental risk for fish populations.

In conclusion, here we present novel although preliminary data on an immune–thyroid interaction in trout. The presence of THRs, particularly the greater expression of THRA in immune organs and cells, suggests that these tissues are responsive to THs. The faster and greater changes in THR expression in immune cells under T3/PTU treatment compared to organs (i.e.: liver) indicates that the effects are particularly noticeable in this system. In addition, the alterations of molecular markers of immune cells subpopulations after T3 and PTU treatment support this hypothesis.

Acknowledgments

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IV. GLOBAL DISCUSSION

1. ENVIRONMENTAL MONITORING OF LOW LEVELS OF POLLUTANTS BY A COMBINATION OF BIOLOGICAL AND ANALYTICAL TECHNIQUES

In nature, organisms are continuously exposed to complex mixtures of pollutants which are usually present in trace or low concentrations (in the ng/g or ng/l range). These pollutants have a very diverse origin and as such, they present particular physico-chemical properties which will determine their fate and toxicity. The sources of exposure are also varied, including for instance, water, food and air, with farmed animals being additionally exposed to other compounds such as antibiotics and steroid hormones that could reach them through the feed or even being generated by themselves (for instance steroids that are released to the water). Considering the diverse sources of exposure and the large amount of potentially deleterious compounds reaching the environment, together with the fact that new chemicals are continuously developed and released, the task of identifying and quantifying them is not an easy one. In this multi-pollution context, the development of new monitoring approaches and techniques is of paramount importance.

Trying to meet this end, we have developed and followed different monitoring approaches which combine both biological and chemical techniques with the ultimate aim of detecting very low levels of pollutants and their effects on fish. Here we summarize and discuss the main findings obtained in Papers I (Quesada-Garcia et al. 2015) and II (Quesada-Garcia et al. 2013).

1.1. Biological monitoring

1.1.1. Monitoring pollution with in vitro bioassays

In vitro bioassays are becoming increasingly popular tools in environmental monitoring (Bolsc et al. 2005; Bury et al. 2014; Segner 2004). In addition to their cost-effectiveness and rapidness, they allow sensitive high-throughput screening and provide an accurate measure of mixture interaction. This is, they account for the overall biological activity including that of potential transformation products and usually present low detection limits (Leusch et al. 2014).

Hydrophobic organic compounds such as PCBs, PBDEs and PAHs among others, exhibit high toxicity and persistence and unfortunately are ubiquitous in the environment (Qiao et al. 2006). Due to their hydrophobic nature they are deposited and accumulated in sediments and may biomagnify along the food chain (Khairy et al. 2014). The effects of dioxin and dioxin-like

compounds are mediated by the aryl hydrocarbon receptor (AhR) and therefore, their presence in a given environmental sample can be detected *in vitro* on primary cultures or permanent cell lines expressing functional forms of CYP and AhR. The usefulness of this approach in ecotoxicological hazard assessment has been demonstrated for a number of complex environmental matrixes including sediments (Qiao et al. 2006), wastewater influents and effluents (Dagnino et al. 2010; Smital et al. 2011), and drinking water samples among others (Wang et al. 2014b). In this study described in paper I, we employed the rainbow trout gonadal cell line RTG-2 to detect EROD inducers in sediments collected along a scarcely impacted river located in the Community of Castile-Leon. In total, seven sampling sites in points of interest such as before and after towns or waste water treatment plants (WWTPs) were selected.

Here, all sediments but one provoked a dose-dependent induction of EROD activity, which allowed us to calculate EC_{50} values, ßNF-equivalents and therefore to rank the sites according to their degree of pollution. Bio-equivalents (BEQs) have been defined as the concentration of a reference compound that exhibits the same effect as the analyzed sample (Wagner et al. 2013). In our case, we have used ßNF-equivalents. They are undeniable useful in quantifying the effects of complex samples and have been applied in many environmental studies (e.g.: Boronat et al. 2009; Qiao et al. 2006). However, the selection of the mathematical model appears to be a critical point which will determine the accuracy of the result. In general, three main models are used to calculate BEQs: linear interpolation, nonlinear interpolation and the ECx model, each of them with particular advantages and limitations (Discussed in Wagner et al. 2013). We decided to use an interpolation approach, using the sigmoidal curves representing the increase in answer with the increase in the concentration of the reference compound (ßNF) and the problem sample (sediment), i.e. the dose-response curves, and determining from the corresponding mathematical equations the sample concentration that provokes the same response as the EC_{50} of the reference compound (ßNF). Although TCDD is the prototypical AhR ligand and therefore commonly used as reference or positive control when calculating toxicity equivalents (as TCDD equivalents, TEQs), here we have preferred to use the AhR agonist βNF, which is much less toxic and easier to handle. The calculated βNF-equivalents of the environmental samples were in the range of ng/mg sediment, which is comparable with results obtained in previous works (Boronat et al. 2009; Puy-Azurmendi et al. 2010). More specifically, (Boronat et al. 2009) analyzing sediments from remote mountain lakes, located far away from direct human impact found βNF-equivalents ranging from 0.35 to 1.5 ng/mg sediment. (Puy-Azurmendi et al. 2010)on the other hand, analyzed sediments from the Urdaibai stuary, a UNESCO biosphere reserve impacted by several anthropogenic activities (agricultural, industrial)

and exhibiting relatively high levels of PAHs. In that study, βNF-equivalents ranged between 0.1 and 0.9 ng/mg sediment (Puy-Azurmendi et al. 2010). The lower βNF-equivalents found in those studies compared to ours may be partially explained by the fact that they employed an AhR-based recombinant yeast assay, probably less sensitive than the RTG-2 system. In fact, the EC_{50} value for βNF was 40 μg/L, ten times higher (Boronat et al. 2009)than the EC_{50} value here reported.

It is interesting to note that the βNF-equivalents are always higher in those sediments collected downstream the towns, indicating that despite the small number of inhabitants, the impact is high enough to be detected by our cellular system. Samples collected downstream the WWTPs (SS4 and SS6) exhibited similar or higher βNF-equivalents than the corresponding samples collected upstream (SS3 and SS5, respectively), indicating the limited capacity of those particular WWTPs to remove AhR-agonists from water. In fact, previous works have reported AhR-agonistic activities in WWTPs effluents (Dagnino et al. 2010; Rao et al. 2013). These studies have highlighted that the type of treatment technology employed in the WWTP is critical in removing such substances given that some systems are quite inefficient.

1.1.2. Biomarkers

Biomarkers constitute another cost-effective method of performing environmental monitoring. These have been defined as physiological or histological changes that are indicative of exposure to or effects of xenobiotics at the organismal (or suborganismal) level (Mayer et al. 1992). They are valuable tools in ecotoxicology and environmental monitoring and have even been proposed from a regulatory perspective as they are integrative tools which may answer Water Framework Directive´s (WFD) challenges (Sánchez and Porcher 2009). Their value resides on their rapidness, cost-effectiveness and sensitivity, which makes them very useful as early-warning systems. Among the wide range of biomarkers, induction of mixed-function oxidases such as cytochromes CYPs are commonly employed. CYP enzymes are involved in reactions related to biotransformation of endogenous and exogenous substances and are rapidly induced in the presence of a wide variety of compounds. More specificallm y, CYP1A and its associated enzymatic activity, named EROD, are sensitive and well established biomarkers in fish and have proven useful in hundreds of environmental studies (Rev. in van der Oost et al. 2003; Whyte et al. 2000). Traditionally, environmental biomonitoring using fish has involved the collection of feral fish by e.g. electrofishing (Maier et al. 2014) or their caging (Brammell et al. 2010). The first strategy has several disadvantages, including the ability of fish to migrate or the generation of

resistance mechanisms (Brammell et al. 2004; Wirgin et al. 2011). Concerning the second method, it involves the maintenance of fish in reduced spaces, which can be an additional source of stress and may interfere with the study (Oikari 2006). Moreover, when a high number of points are to be monitored with a relatively high frequency (e.g.; monthly etc), the search of more cost-effective solutions is required. In this way and as an alternative, we propose the use of fish farms as tools to regularly monitor trace levels of contaminants in water.

In our first study (Paper I), we selected a rainbow trout fish farm located downstream the river whose sediments were subject to the cellular assays (Farm A), and juvenile females were sampled monthly. Parallel samplings of animals with similar conditions (same species, sex, age and approximate weight) were performed in another farm located in Castile-La Mancha (named B) with the aim of obtaining reference values and avoid season-related or any other confounding factor. Farm A exhibited EROD values ranging from 131 ± 15 to 387 ± 39 pmol/mg/min. These were much higher at all sampling times compared to farm B, in which values ranged from 22 to 85 pmol/mg/min. These values measured in farm A do not fall within the physiological range described for rainbow trout microsomes which is defined between 4 and 100 pmol/mg/min (Brammell et al. 2010; Fenet et al. 1998; Gourley and Kennedy 2009; Jonsson et al. 2006). This EROD induction in farm A was accompanied by an overexpression of *cyp1a* mRNA levels. However, these increases were significantly different (p<0.05) with respect to the other studied months only in September and October, probably due to the variability of the results, consequence in part of the relatively limited amount of data (n=8).

In addition, transcriptional levels of another cytochrome named CYP3A and implicated in detoxification processes were also assessed. *Cyp3a* was overexpressed at all sampling times, although the trend was different to that exhibited by *cyp1a*, suggesting that the inductions were provoked by different substances or mixtures of substances. Although previous studies have associated EROD and *cyp1a* inductions with antiestrogenic effects, both *in vitro* (Navas and Segner 2000) and *in vivo*, (Valdehita et al. 2012), here we found no association and therefore will not be further discussed.

Our second study (Paper II) is actually a continuation of the previous work. We started by performing routine samplings in the fish farm located in the Autonomous Community of Castile-La Mancha (previously named B). This farm is located in an isolated area, far from any industrial activity or anthropogenic pressure and has always shown basal EROD activity values (Quesada-Garcia et al. 2015; Valdehita et al. 2012). In total, 5 monthly samplings corresponding to the period comprised between October 2011 and February 2012 were analyzed. In addition,

six months later, in September 2012 an additional sampling was performed. In each sampling, CYP1A and CYP3A were evaluated at the enzymatic and transcriptional levels. In the first two samplings (October & November 2011) EROD activity values were below 100 pmol/mg/min, which falls within the range considered as physiological (Brammell et al. 2010; Fenet et al. 1998; Gourley and Kennedy 2009; Jonsson et al. 2006). However, in the subsequent months, and without apparent reason, EROD activity experienced an induction, which reached a maximum in February 2012 (541 pmol/mg/min). Six months after this peak, we sampled again to verify whether the situation had improved. EROD continued to be high although lower than in February (254 ± 56 pmol/mg/min). The inductions here observed, specially the peak measured in February 2012 are comparable to others reported previously. For instance, rainbow trout injected with βNF also exhibited a strong EROD induction with levels ranging between 600 and 800 pmol/mg/min (Fenet et al. 1998). More recently, juvenile rainbow trout receiving 5.04 mg/L of the fungicide propiconazole (Li et al. 2013), 25 µg/L benzo(*a*)pyrene or 3.26 µg/L PCB-126 (Jonsson et al. 2006) exhibited hepatic levels ranging between 500 and 800 pmol/mg/min. In addition to chemicals, EROD activity may be induced by a number of natural substances. As an example, humic substances (HS), which are organic molecules present in most freshwater systems are known to modulate biotransformation processes including induction of EROD activity (Andersson et al. 2010; Matsuo et al. 2006). In this regard, several facts must be highlighted. In the first place, (Andersson et al. 2010), exposing three-spined stickleback to different concentrations of both natural and synthetic HS found gill but no hepatic EROD induction. (Matsuo et al. 2006) found hepatic EROD activity in tambaqui (*Colossoma macropomum*), an Amazonian species after exposure to HS. However, it must be noted that those effects were observed when tested at a concentration of 20-80 mg C/L, well above the mean concentration found in most freshwaters around the world (0.5- 4 mg C/L)(Thurman 1985). Although particular ecosystems such as the Amazon basin may exhibit much higher levels of HS (>50 mg C/L) and therefore affect the results of EROD activity, in our conditions the impact of such compounds is not that likely.

In this study, we also decided to include another enzymatic activity, named BFCOD and associated to the CYP3A. Compared to CYP1A and EROD activity, CYP3A and its corresponding enzymatic activity BFCOD, have been much less studied. In fact, while a search in Pubmed (www.ncbi.com) with the terms “CYP1A OR EROD” AND “Fish” retrieves 1574 citations, the corresponding search with CYP3A and BFCOD recovers only 144, which complicates the task of establishing basal levels and comparing our results with other authors. Here, in the first 5 samplings, BFCOD levels ranged from 7 to 26 pmol/mg/min, which according to the relatively

scarce literature can be considered as basal (Hasselberg et al. 2008; Hegelund et al. 2004; Wassmur et al. 2010). Surprisingly, six months later, in September 2012, BFCOD reached a maximum (102 pmol/mg/min). Up to our knowledge, only one work has actually assessed BFCOD activity in a field study (Della Torre et al. 2010). In that work, BFCOD was measured in livers of red mullet *(Mullus barbatus)* collected from three different sampling sites showing varying degree of PAH contamination. BFCOD activity was approximately 2 and 3.5 times higher in the high impact site compared to the moderate impact and reference site, respectively. The fact that no other study has assessed rainbow trout BFCOD under field conditions, together with BFCOD basal levels varying widely across species (Ribalta and Sole 2014), complicates the comparison of the absolute values and therefore the interpretation of our results. On the other hand, recent evidence has suggested that although in mammals BFC is a CYP3A-specific substrate, in zebrafish it can be metabolized by other CYPs including CYP1A (Scornaienchi et al. 2010). More recently, a study has found that fish BFCOD activity is highly induced by several prototypical AhR ligands both *in vivo* and *in vitro* (Creusot et al. 2014). These data altogether suggests that BFCOD could be used as a non-specific marker of phase I metabolism. In any case, it must be noted that in our study EROD-BFCOD, and CYP1A-CYP3A did not follow the same induction patterns, indicating that substances (or mixtures of substances) provoking the inductions were not the same and that these enzyme activities and CYPs follow different induction patterns.

1.1.3. Active biomonitoring: an useful tool in environmental monitoring.

After the 3-fold and unexplained increase in EROD activity in February 2012, we decided to follow an active biomonitoring (ABM) approach in which 16 rainbow trouts from the studied farm were transferred to a clean farm in the Polytechnic University of Madrid (Superior School of Forest Engineers, Madrid, Spain). This approach consists on the translocation of organisms from one place to another (from clean to polluted sites or vice versa) in order to compare their responses at different levels (e.g.: biochemical, physiological, etc) and, combined with other techniques such as biomarkers, has proved useful in a number of environmental studies (He et al. 2011; Wepener et al. 2005). In those studies, animals were transferred from reference to polluted sites but here we preferred to follow the inverse procedure in order to assess the ability of recovery.

After only seven days in clean waters, EROD activity dropped to less than 25% of the original values, practically recovering the initial levels recorded in October and November 2011. At the same time, BFCOD activity also decreased by half, recovering the values observed in

October. In September of the second year, when BFCOD activity was maximal, another ABM experiment was carried out. This time we decided to extend the time of the experiment to 30 days. EROD activity experienced a decrease to 20% of the original values after 7 days although additional maintenance for 15 and 30 days did not provoke further decreases. BFCOD activity was also reduced by almost half after only one week (60 ± 17 pmol/ mg protein/ min). Additional maintenance for 15 and 30 days produced a further significant (p<0.05) decrease (reaching 26 ± 7 pmol/mg protein/min and 18 ± 12 pmol/mg protein /min, respectively) in BFCOD values with respect to the initial ones. These observations at the enzymatic level were also accompanied by decreases in *cyp1a* and *cyp3a* mRNA levels.

The strong decrease in both enzyme activities and mRNA levels after transferring the fish to clean water is consistent with the notion that the fish had been exposed to contaminants. Interruption of this exposure may have allowed the catabolization or elimination of the responsible compounds (Brammell et al. 2010), leading to the reduction or even disappearance of the detoxification responses. The fast recovery of basal levels in several biomarkers after transference to clean waters has been previously demonstrated in few studies. For instance, juvenile rainbow trout exposed to benzo(*a*) pyrene (BaP) showed an EROD induction similar to that here reported in February 2012 (450 pmol mg prot min). Maintenance in clean water for 14 days provoked a progressive recovery of basal levels of EROD activity (Jonsson et al. 2006). In another study in which three-spined stickleback were collected from a polluted river and transferred to clean water and maintained for 15 days, EROD levels decreased by an 83% (Sanchez et al. 2007). That decrease is comparable to that found in our study.

Regarding BFCOD activity, up to our knowledge no other study has assessed the recovery of basal levels after transfer to clean waters, hindering the comparison of results. However, despite the lack of reference values, it must be noted that in this study BFCOD decreased almost by an 82% (from 102 until 18 pmol) indicating that the values measured at day 0 were indeed highly induced.

1.2. Chemical monitoring

Chemical analyses are widely used in environmental monitoring and especially accepted from a regulatory perspective (Leusch et al. 2014). However, the main and evident disadvantage is their limited utility in toxicity assessment as they provide no information on the potential deleterious effects on biota (Leusch et al. 2014; Qiao et al. 2006). In addition, chemical analyses are not able to evidence any possible interaction (additive, synergistic, antagonistic) among

compounds. Finally, they cannot show the presence of a plethora of substances (hundreds or thousands) below detection limits, substances that can interact among them and provoke strong deleterious effects while being unnoticed in the analyses.

1.2.1. GCxGC-TOF-MS

The very same sediment samples analyzed by means of the *in vitro* assay were subjected to chemical analyses with a GCxGC-TOF-MS. Analyses demonstrated the ubiquitous presence of low concentrations of PAHs in the ng/g dw range. Given that each PAH exhibits different toxicity, total TEQs (2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalents) were calculated by multiplying each concentration in the mixture by the corresponding individual toxic equivalency factor (TEF) (Van den Berg et al. 2006). Samples exhibited low TEQs (0.16- 2.17 pg TEQ/g dw), similar to that obtained in previous works (Valdehita et al. 2012) and well below the safety limit of 20 pg TEQ/g dw (Evers et al. 1996). Several personal care products (PCPs) were also detected in the samples in the same range, indicating that they are good markers of anthropogenic impact.

In our second study (Quesada-Garcia et al. 2013), we decided to analyze samples from the entry channel of the fish farm. Those samples were taken monthly, at the same time that those of fish, and analyzed by GCxGC-TOF-MS. Analyses also evidenced ubiquitous presence of PAHs in the ng/g dw range. Pyrene and fluoranthene were found at a considerably higher concentration in February 2012, when the maximal EROD induction was found. Specifically, the concentration of pyrene was between 100 and 275 and fluoranthene was between 11 and 200 times higher than in the other months. In order to support this discussion, TEQs were calculated in the sediment samples of each of the four months subject to study (these results are not shown in Paper I and have been calculated *a posteriori).* TEQs in sediments (pg/g dw) were: 7.44 (October 2011), 4.14 (November 2011), 44.45 (February 2012) and 2.05 (September 2012). Sediment samples from February 2012 exhibited a value of 44.45 pg/g dw, which is above the safety limit of 20 pg TEQ/g dw (Evers et al. 1996). Even though these two PAH do not induce EROD activity *in vitro* (Behrens et al. 2001; Bolsc et al. 1999; Quesada-Garcia et al. 2015), it is widely accepted that PAHs usually do not appear alone in sediments but as mixtures of hundreds of related compounds (Neff et al. 2005). In this way, the simultaneous presence of those two compounds in a considerably higher concentration together with the high value of TEQ obtained, could serve as an indicator of an episode of isolated contamination involving a variety of PAHs and related compounds which may explain the strong EROD induction measured in that month.

1.3. Chemical vs. Biological data: Considerations

1.3.1. Correlation between chemical and in vitro bioassay analysis (Paper I).

In order to investigate any association between calculated βNF-equivalents (derived from biological assay with RTG-2) and TEQs (calculated from chemical analysis), a Pearson correlation analysis was performed. Interestingly, and despite the low number of data (7 points), a very good positive correlation was found($r=0.840$, $p<0.05$). This finding however, does not imply that the observed inductions are provoked by those chemicals. In fact, when the four PAHs and three PCBs whose concentrations served to calculate TEQs were assayed (either alone or in combination) in the RTG-2 system, they failed to induce EROD. What does this mean then? A possible explanation is that the observed inductions are caused by other co-occuring substances which are i) not identified, ii) present in undetectable concentrations, iii) or interacting among them (additive, synergistic, antagonistic). In fact, although chemical analyses are undeniably useful, their main disadvantage in environmental monitoring is precisely that usually only chemicals for which standards and techniques are available can be identified and quantified (Wang et al. 2014a). Moreover, substances present at very low concentrations, below de detection limit, will pass unnoticed and their possible effects will not be taken into account. In addition, chemical analyses are not able to reflect additive, synergistic or antagonic interactionss amongst detected or undetected chemicals. Therefore, chemical analysis are not an appropriate tool for reflecting possible biological (toxic) effects.

1.3.2. General considerations: learnt lessons

Environmental monitoring is probably one of the biggest challenges within the field of Ecotoxicology. In comparison with laboratory-based studies, in which controlled conditions can be set, the plethora of factors affecting the results of field studies complicate the task of establishing causal-effect relationships. Despite the enormous advances in technology and knowledge experienced in the last decades, up to now no single monitoring method fulfills all the necessary requirements nor answers all the posed questions. Chemical analyses are useful in identifying and quantifying pollutants but provide no information on the potential deleterious effects on biota. On the other hand, biological monitoring, valuable in observing differences between polluted-pristine environments, does not inform about the pollutants causing such effects. In addition, organisms in nature are subjected to a series of factors (e.g.: temperature, season, genetic variability, etc) which may affect the responses and confound the conclusions. However, the appropriate use of the information obtained by applying biomarkers can give very

valuable data about the kinds of pollutants responsible of the alterations observed in organisms. In this way and taking into account the advantages and limitations of each method, the best approach would probably be to combine multiple lines of evidence, as suggested by other authors (Leusch et al. 2014; Zounkova et al. 2014) in trying to avoid over or understimations of the risks. In particular, the use of an active biomonitoring approach, has proved very useful in our study to demonstrate exposure and recovery. Future studies should further explore the possibilities (and limitations) offered by this cost-effective strategy.

2. PRESENCE OF ENDOCRINE ACTIVITY IN FISH FEEDS

In addition to water, feeds can be another potential source of endocrine disruptors and other contaminants for farmed fish (Berntssen et al. 2010; Mantovani et al. 2009). Several studies, analyzing commercial pellets commonly employed in aquaculture, have reported the presence in feed pellets of high concentrations of environmental pollutants such as PCBs, PAHs, PBDEs and organochlorine (OC) pesticides among others (Easton et al. 2002; Hites et al. 2004; Serrano et al. 2003). In addition to such environmental pollutants, it is well established that pellets can contain steroids and steroid-like compounds (Pelissero and Sumpter 1992). These include for instance actual hormones, synthetic chemicals and natural compounds of vegetal origin such as phytoestrogens (Pelissero and Sumpter 1992).

Commercial fish feeds are complex mixtures of different ingredients from both animal and vegetal origin. Fish meal, which is usually the main animal ingredient and represents 15-40% of the diet in weight, is prepared from whole animals, including glands and sexual organs, and as a consequence, may contain both estrogens and androgens although the type and amount of steroids is quite unpredictable (Pelissero and Sumpter 1992). Although the contents of these substances would be similar to those found in wild fish serving as food, some concerns could raise in case that they appear in particularly high concentrations in fish meal. On the other hand, the rapid increase in intensive aquaculture accompanied by an increase in aquafeed production has urged to find vegetal replacements with the primary aim of ensuring the long-term sustainability of the aquaculture industry (Gatlin et al. 2007). However, vegetable meals used as substitutes also contain EDs, mostly phytoestrogens. Soybean meal for instance, commonly employed in animal diets, has shown to contain a high amount of two estrogenic isoflavones, daidzein and genistein (Pelissero and Sumpter 1992). In addition, a third potential source of EDs exposure to fish through the food are certain pesticides and environmental pollutants that could be present in the pellets. Some of them such as the weak estrogenic pesticide DDT (Wetterauer et al. 2012), and its metabolites have been found in fish feeds in the ng/g range (Jacobs et al. 2002; Maule et al. 2007). Overall, this means that the type and concentration of potential hormonally-active substances is very varied what complicates the task of identifying and quantifying such compounds. Similarly to other environmental samples such as sediments, fish feeds are very complex matrices in which synergistic, agonistic and antagonistic effects among substances may pass unnoticed if only chemical analyses data are considered. As an alternative, the use of *in vitro* bioassays is a rapid, cheap and powerful tool to characterize the potential endocrine activity of feed pellets. Although they do not provide precise information on which

pollutants are present and on the molecular targets of such compounds, they allow to semi-quantify the total hormonal load of a given sample.

In this context, chapter 2 describes a study whose purpose is to determine the total estrogenic, thyroidal and AhR-agonistic activity load of a set of commercial fish feeds. While some studies have already posed the question of whether feeds represent a source of estrogenic substances (Matsumoto et al. 2004), up to our knowledge this was the first study assessing their potential thyroidal activity *in vitro.*

2.1. AhR-agonistic activity of commercial fish feeds.

It is nowadays recognized that fish origin products such as fish oils can contain relatively high levels of persistent organic pollutants including PCBs, polychlorinated dibenzo-p-dioxins/polychlorinated dibenzofurans (PCDD/Fs) and other dioxin-like compounds. Provided the hydrophobic nature of those compounds, they may bioaccumulate along the food chain and pose a threat to organisms situated on top. As a consequence of the increasing concern, several methods have been developed to monitor the presence of such compounds in animal products (Malisch and Kotz 2014; Parera et al. 2013). Traditionally, samples are analyzed by means of high-resolution gas chromatography/high-resolution mass spectrometry (HRGC/HRMS) but the method does not allow high-throughput screening and bio-analytical methods have arisen as an alternative (Hasegawa et al. 2007; Stypula-Trebas et al. 2009).

In order to test the presence of AhR-like compounds in fish feeds, we evaluated EROD activity induction in the RTG-2 cell line, following an approach similar to that discussed in section 1.1.1. In a preliminar experiment, we selected 21 fish feeds, among which 17 induced EROD activity in at least one of the concentrations tested (Table 1). Maximum EROD activity in those samples ranged between 5 and 66 pmol/mg prot/min, which is equivalent to the response provoked by 0.003 and 0.021 µM of the positive control, BNF. In this set of experiments, the EC50 of BNF was 0.036 µM, meaning that sample 16 (F16) was able to provoke a response in the same order of magnitude to that value. However, further extractions and experiments showed a decrease in the activity, suggesting the instability of the compounds responsible for the activity observed. This lack of repetition, together with the fact that most of the samples did not show a full dose-response curve, prevented us from extracting solid conclusions and we decided not to further continue.

Table 1 Maximal EROD activity measured in RTG2 cells

Sample	Concentration inducing maximal EROD activity (mg fishfeed/ml)	Maximal EROD activity (pmol/mg prot/ min)	Equivalence to BNF response (µM)
F0	8.4	19	0.005
F1	8.4	30	0.008
F2	8.4	13	0.004
F3	8.4	40	0.0113
F4	8.4	14	0.005
F5	8.4	-	-
F6	8.4	-	-
F7	8.4	9	0.004
F8	8.4	10	0.004
F9	8.4	-	-
F10	8.4	-	-
F11	8.4	5	0.003
F12	8.4	35	0.010
F13	8.4	25	0.007
F14	8.4	66	0.021
F15	8.4	15	0.005
F16	8.4	8	0.004
F17	8.4	8	0.004
F26	8.4	5	0.003
F29	8.4	5	0.003
F32	8.4	5	0.003

2.2. Development and validation of HER-LUC cell line

The increasing concern on ER-mediated endocrine disruption experienced in the last decades has resulted in the development of a great variety of *in vitro* assays, whose main advantage is their suitability for large-scale screening (Ackermann et al. 2002). For instance, the E-screen, an assay developed to identify estrogenic environmental compounds, employs the proliferation of human MCF7 breast carcinoma as a marker of estrogenicity (Soto et al. 1992). The yeast screen assay (YES) (Arnold et al. 1996; Routledge and Sumpter 1996), in which the human estrogen receptor (hER) is expressed in yeast, has also been widely applied. In addition, cell lines stably or transiently transfected with the ER and a reporter gene are useful to identify receptor agonists and antagonists (Gray et al. 2002). This last approach has been chosen by many authors (e.g.: Ackermann et al. 2002; Balaguer et al. 1999; Cosnefroy et al. 2009) and is the kind of assay used in the only currently approved Organization for Economic Cooperation

and Development (OECD) test guideline to determine and characterize the estrogenicity of chemicals through *in vitro* approaches (OECD 2009).

When developing a reporter gene assay based on stably transfected cells several facts must be taken into account. First, one evident advantage of the use of stably transfected cell lines is that they do not require repeated transfections and consequently, the assay gains in swiftness, performance and reliability. Since cells are already available to performing the assay without any preliminary transfection experiments there is an important spare of effort and time, and the likelihood of variations is lower compared to transient transfections (Gray et al. 2002). Secondly, cross-species differences in ER binding by environmental chemicals, probably due to the variability in the amino acid sequence in the ligand binding domain (LBD), have been widely reported (Dang 2010). For instance, rainbow trout estrogen receptor (rtER) has shown to exhibit greater ligand promiscuity tan human, murine, avian and reptile ERs (Matthews et al. 2000) but lower sensitivity compared to hER (Le Dréan et al. 1995). This means that in order to characterize estrogenic activities of chemicals, environmental or food samples that could be relevant for fish it is more adequate to use systems comprising a piscine ER. Thirdly, ER-subtype differences in xenoestrogen binding, probably explained by the dissimilarities in the N-terminal and in the LBD (Matthews and Gustafsson 2003), have also been reported (Rev. in Delfosse et al. 2014). In fact, it has been stated that this difference in binding affinities between ER subtypes is bigger than the difference among interspecies ERs (Dang 2010). In spite of these differences in binding affinities, up to date the subtype considered pertinent for screening and testing of EDCs has not yet been specified (Dang et al. 2011). It has been shown that the ligand binding pocket of ERα is bigger than that of the ERβ, suggesting that the former might be more promiscuous, and therefore more appropriate for *in vitro* assays, avoiding false negatives (that in the first tiers of a testing strategy should be preferentially avoided).

In the study presented here and considering all these facts, we developed and validated a reporter gene assay, named HER-LUC, based on the stable expression of the sea bass *(Dicentrarchus labrax)* estrogen receptor α (sbERα) (Muriach et al. 2008) into the human embrionic kidney (HEK-293) cell line. Luciferase (*luc*) gene was used as a reporter gene under the control of estrogen responsive elements (EREs). The use of sbERα is of particular interest and was chosen taking into account that sea bass is a carnivorous species of great importance for Mediterranean aquaculture.

Prior to the evaluation of the fish feed samples and in order to test the proper functioning of the system, the assay was validated using E2 together with soft agonists of the ER

(17α-estradiol, 17α-methyltestosterone) and an ER antagonist (tamoxifen). The selected test compounds have been proposed in the OECD Test Guideline 455 (OECD, 2009) as reference chemicals in *in vitro* estrogenicity studies. As expected, E2 was the most potent and efficient estrogenic compound (EC50= 32nM). EC_{50} values in the nM range have been reported in previous reporter gene assays employing rainbow trout ERs (Ackermann et al. 2002; Cosnefroy et al. 2009; Matthews et al. 2000). However, in those studies EC_{50} values were lower, ranging between 0.5 and 3.3 nM, and therefore indicating that sbERα may exhibit a lower binding affinity for estrogens and estrogen-like substances. Nevertheless, more experiments focusing on binding affinity would be required in order to verify this hypothesis.

In terms of potency, E2 was followed by 17α-estradiol, which was approximately 100 times weaker (EC_{50}= 2.854 μM). The relative agonistic activity (RAA), which is the ratio between the EC_{50} values of E2 and that of the compound of interest, allows the ranking of chemicals according to their potency. In this way, the RAA of 17α-estradiol was 0.01, which is in good agreement with previous studies (Sonneveld et al. 2006). 17α-methyltestosterone turned out to be a much weaker agonist (EC_{50}= 380μM). The antagonist tamoxifen did not induce luciferase activity but inhibited the maximal response induced by E2 in a dose-dependent manner, which indicates that the estrogenic response is specifically mediated by sbERα. Although tamoxifen has been reported to act as an agonist or antagonist in a tissue-dependent manner (Paech et al. 1997), here we did not observe any estrogen-like activity. Our results are in agreement with other authors employing a reporter assay with a piscine ER (Ackermann et al. 2002), suggesting species-specific differences in the response of different organs or tissues to tamoxifen.

2.3. *In vitro* estrogenicity of commercial fish feeds

In this study we assessed the potential estrogenic activity of 32 commercial fish feeds commonly used in aquaculture. In order to detect both polar and non-polar compounds, two different extractions, using methanol and hexane respectively, were assayed. Extracts obtained with hexane did not provoke any response in the HER-LUC cell line. Regarding the methanol extraction, 11 out of the 32 tested pellets (34%) induced sbERα-mediated transcriptional activity although only one induced a full dose-response curve. Relative transactivation activity (RTA) of a given sample is calculated by normalizing its maximal luciferase induction with respect to that produced by the positive control. In our case, the positive control corresponded to a fish feed spiked with 0.25μM of E2 and subjected to the extraction process. In this study, RTAs of the fish

feeds were rather low, ranging between 1.05 and 6.05% and indicating therefore, a weak estrogenic activity.

Estrogenicity caused by commercial foodstuffs destined to a variety of species including fish (Beresford et al. 2011; Matsumoto et al. 2004; Miyahara et al. 2003), reptiles (Miyahara et al. 2003), rodents (Boettger-Tong et al. 1998) and humans (Behr et al. 2011) has been previously described. In the case of fish, it has been shown to severely impact piscine reproductive physiology (Pelissero and Sumpter 1992). The reported estrogenicity in foodstuffs has been mostly explained by the presence of high concentrations of phytoestrogens such as genistein and daidzein as revealed by chemical analyses (Boettger-Tong et al. 1998; Miyahara et al. 2003). In fact, (Kaushik et al. 1995) found high concentration of glucuronides of daidzein and genistein in the bile of trout fed soy flour rich diets, and attributed the poorer growth rates to these substances. The retardation effect of estrogens and xenoestrogens on growth parameters and its correlation with reproductive endpoints has been observed in a number of species including brown trout (*Salmo trutta*) (Schubert et al. 2014), zebrafish (Schafers et al. 2007) and fathead minnow *(Pimephales promelas*, (Länge et al. 2001). This may be resulting from energetic constraints, as the metabolism of exogenous estrogens would limit the energy accessible for growth or reproduction (Burkhardt-Holm et al. 2008). In addition, some evidences suggest that estrogens may modulate the growth hormone (GH)/insulin-like growth factor I (IGF-I) system (Shved et al. 2008).

Of course, results obtained with *in vitro* approaches are difficult to translate to *in vivo* situations due to differences in metabolic capabilities and the lack of the whole regulatory and feed-back processes acting in whole organisms, however they give important information about mechanisms that can be of relevance. Although estrogenic effects caused by the diets studied in this work were moderate and in some cases actually very low or inexistent it must be taken into account that these food pellets are administered continuously and that doses fed daily by fish can reach yearly 10^3 to 10^5 times those reported here depending on fish size and food intake level. Therefore, the results obtained with this *in vitro* approach should be considered as an early warning of possible effects observed in fish farms at long term.

2.3. *In vitro* thyromimetic activity in commercial fish feeds.

Regarding the disruption at the thyroid-receptor level, it was screened with an already established reporter gene assay, named PC-DR-LUC, which expresses luciferase gene under the control of avian (av) THRα1 (Jugan et al. 2007). Ideally, the assessment should have been performed with a cell line expressing the receptor of a piscine species. However, unfortunately at the time of the experiments no such commercial cell line was available and our attempts to generate it were not successful. Similarly to ERs, differences in receptor binding affinities or receptor-DNA interactions must be considered. For instance, rainbow trout nuclear thyroid receptor showed a lower affinity for T3 compared to other animal species, including chicken. On the other hand, when receptor-DNA interactions were compared, receptors from dog and rainbow trout liver were similar (Ichikawa et al. 1989). However, according to (Jugan et al. 2007) there are no major discrepancies between the ligand, DNA, and co-factor binding properties of the avian THRα1 and that of the mammalian receptor. In a more recent study, (Oka et al. 2013) established several transient transactivation assays using THRs (α and β) from 3 frogs, one fish, one alligator and human and compared the cross-species differences. Although the study demonstrated some species-specific differences, similar transcriptional activities were generally found in all species examined. For instance, the activation responses using human THRs transfected in the mammalian cell line HEK-293 was the same as that of the piscine THRs transfected into the same cell type (Oka et al. 2013). In those transient assays, T3 exhibited EC_{50} values in the same order of magnitude to those reported for PC-DR-LUC (10^{-10} M), indicating the reliability of this assay. In any case and despite of these differences, the use of an already established assay, as a first approach in assessing potential thyroid disruption in fish feeds, was considered the correct option. Future approximations should include the development of a cell line transfected with a THR from a fish species (e.g. rainbow trout or sea bass), not described to date.

To the best of our knowledge, our work is the first to report the detection of thyroidal activity in fish feeds. Here, again hexane extracts had no effect on the reporter activity while 56% of the methanol-extracted pellets (18 out of 32) had thyromimetic effects. In this case, RTA values were considerably higher, ranging between 9.96 and 47.54% and indicating a strong hormonal activity.

In comparison to estrogenicity, much less work has focused on the potential thyroidal activity of feeds on fish physiology and in light of our results two questions undoubtedly arise: i) what substances present in the foodstuffs are responsible for the observed inductions and ii)

which effects are to be expected in the long term in farmed fish eating continuously these pellets. Regarding the first question and taking into account the absolute lack of literature on the topic, it is very difficult to answer without performing chemical analyses. We could speculate that given that the extracts performed with hexane did not provoke any induction of the THR-mediated luciferase activity, thyroidal activity found in the fish feeds may be mainly due to polar compounds including hormones and some non-hydrophobic pollutants. In addition, only 5 samples caused both ER and THR transactivation, suggesting that inductions are provoked by different substances. Nevertheless, more specific analyses should be done for confirming or discarding this hypothesis.

In order to answer the second question (long term effects on fish), also further experiments would be needed. As described in the introduction, thyroid hormones, which are regulated by complex feedbacks and compensatory mechanisms, act as pleiotropic factors. In fish, in addition to growth and development (Rev. in Power et al. 2001), THs are involved in many other functions such as thermal acclimation (Little et al. 2013), pigmentation (McMenamin et al. 2014) and gonadal sex differentiation (Mukhi et al. 2007) among others. In mammals, one target of TH action appears to be the immune system and the existence of a bi-directional and complex relationship between both systems has been widely discussed (De Vito et al. 2011; Klein 2006). On the contrary, much scarcer information exists on whether THs play an immunomodulatory role in fish, which will be the focus of the third part of the present thesis.

3. THYROID SIGNALLING IN THE PISCINE IMMUNE SYSTEM

In vertebrates, the action of thyroid hormones (THs) is multifaceted as they exert pleiotropic functions beyond development and reproduction (e.g.: Grais and Sowers 2014; McAninch and Bianco 2014). Accumulating evidence over the last years indicates that thyroid hormones may also act on the immune system (Arpin et al. 2000). In fact, in mammals, the existence of a complex and bi-directional relationship between the hypothalamus-pituitary-thyroid (HPT) axis and the immune system is generally well accepted (De Vito et al. 2011; Klein 2006). For instance, mammalian white blood cells and mast cells contain T3 (Csaba et al. 2004; Pallinger et al. 2005) and express thyroid hormone receptors (THRs) (Barish et al. 2005; Hastings et al. 1997). THs influence the distribution and proliferation of B and T lymphocytes, cell-mediated immunity, macrophage maturation, etc (De Vito et al. 2012; De Vito et al. 2011; Perrotta et al. 2014). On the other hand, several cytokines (i.e.: il1, il6, tnfa) affect the release of pituitary hormones, suppressing the HPT axis (Haddad et al. 2002). However, in teleost fish this possibility has been scarcely explored (Harris and Bird 2000; Yada and Nakanishi 2002). In fact, while the hypothesis of an immunomodulatory action of estrogens and other hormones has been suggested in fish (Casanova-Nakayama et al. 2011; Milla et al. 2011), only indirect evidence supports such a role for THs.

Considering this lack of knowledge, together with the increasing concern of the worldwide presence of thyroid-disrupting compounds in the aquatic environment, the objective of the study was to shed some light onto the possible impact of THs and thyroid-disrupting compounds on immune system of teleostean fish. In order to do so, the following questions were addressed.

3.1. Is the piscine immune system sensitive to thyroid hormones? Expression of *thra* and *thrb* in immune organs and cells.

The classical mechanism of TH action involves ligand-binding to their corresponding receptors, the THRs. So far, two main subtypes of receptors, THRA and THRB, have been described in all vertebrates including fish. In mammals, the expression of THRs in the organs (Nishimura et al. 2004) and cells of the immune system such as B lymphocytes (Hastings et al. 1997), macrophages (Perrotta et al. 2014) and mast cells (Siebler et al. 2002) among others, is well established but such possibility has not been previously explored in a piscine species.

In order to test if direct signaling of natural thyroid hormones in immune system of rainbow trout is possible, the first step was to explore whether the immune organs and total immune cells of rainbow trout expressed *thrs*. To this end we analyzed *thr* expression in two immune organs, spleen and total head kidney. Compared to mammals, in which the primary site for hematopoiesis is the bone marrow, in teleost fish the head kidney serves this function (Zapata et al. 1996). The teleost head kidney is a complex tissue comprised of immune cells (macrophages, lymphocytes) but also containing other cell types such as hematopoietic (lymphoid cells) and endocrine cells (chromaffin cells, corticosteroidogenic cells) among others. Considering this fact, we decided to isolate pure immune cell populations from this organ by means of a Ficoll-Hypaque® density gradient. The same reasoning was followed for the blood, comprised of erythrocytes or red blood cells (90%) and leukocytes (10%) (Niimi and Lowe-Jinde 1984), and therefore pure immune cells were isolated. Parallely, *thra* and *thrb* levels were measured in the liver as reference organ.

Both receptor subtypes were expressed in all the examined tissues and cells, although expression varied in a subtype and tissue-specific manner. *Thra* showed higher expression in the immune organs and isolated immune cells compared to liver, while *thrb* exhibited the opposite expression pattern. More specifically *thra* was between 7.32 and 11.77 more expressed in immune-related organs and leukocytes than in liver. On the contrary, *thrb* expression was between 1.37 and 7.31 times lower in immune organs and cells than in liver. Our results are in accordance with other studies which also found higher expression of *thra* in human spleen and head kidney compared to liver (Nishimura et al. 2004). In the same way, a higher expression of *thrb* than of *thra* in liver has been described for a number of species including rainbow trout (Raine et al. 2005), rat (Zandieh-Doulabi et al. 2003) and humans (Nishimura et al. 2004). Differences between whole organ and isolated cells from head kidney were also observable. Both *thrs* exhibited higher expression in the whole organ, indicating that they are also expressed in non-immune cells (e.g.: vascular endothelial cells etc). However, a clear disparity was found between subtypes. While *thra* showed similar levels in both organ and isolated cells, *thrb* showed strong differences. These results indicate that *thra* is mostly expressed in immune cells while *thrb* is found mainly in non-immune cells. Tissue-specific expression of nuclear receptors including *thr* has been previously reported (Harada et al. 2008; Kawakami et al. 2003; Liu et al. 2000) and may imply that receptor subtypes serve different functions (Liu et al. 2000). Our results suggest therefore that *thra* is the main subtype of the effects of THs on trout immune cell function. Given that our data refers to total isolated immune cells, which contain different proportions of lymphocytes B and T, macrophages etc., further studies should also evaluate THR

expression in those specific populations of immune cells and assess whether their expression pattern changes under different developmental and physiological conditions.

In any case, the demonstration that THR is expressed not only in immune organs but also in purified leukocyte cells of rainbow trout supports the notion that THR signaling can occur in the immune system of this piscine species.

3.2. *In vivo* effects of T3/PTU

3.2.1. Effects on dio2 expression

After confirming that both receptor subtypes are expressed in the immune system of rainbow trout, we decided to explore whether THR mRNA levels are responsive to the thyroidal status of the organism. In order to do so, we carried out an *in vivo* experiment in which juvenile rainbow trout were exogenously administered (through the food) with the active thyroid hormone T3 or the goitrogenic drug PTU. In order to determine whether the treatments were effective we evaluated changes in the hepatic enzyme deiodinase 2 (*dio 2*). This hepatic enzyme controls the intracellular concentration of T3 (Orozco and Valverde 2005) and is widely used to monitor thyroid status in teleost fish (Johnson and Lema 2011; Mol et al. 1999). The main function of this enzyme is to convert T4 into the biologically active T3 by removing iodine from the 5´ outer-ring site, and has demonstrated to be highly sensitive to changes in circulating hormones (Johnson and Lema 2011). An increase in plasma THs, as expected after exogenous administration of T3 would provoke a decrease in the expression of this gene while PTU would provoke the opposite effect. Here, treatment with T3 led to a 75% decrease in the relative abundance of *dio2* transcripts at both sampling dates, which is in agreement with previous studies. For instance, Nile tilapia (*Oreochromis niloticus*) fed T3-supplemented food for 11 days exhibited a strong decrease in hepatic *dio2* mRNA levels (Mol et al. 1999). In our study, exposure to PTU caused a 3-fold increase in hepatic *dio2* mRNA levels compared to control. Similar *dio2* up-regulation after exposure to PTU has been reported for other piscine species such as seabream (*Sparus aurata*) (Morgado et al. 2009) and fathead minnow (Noyes et al. 2013).

3.2.2. Effects on thra and thrb expression in organs and isolated immune cells.

THR genes contain TREs and their transcription can therefore be autoinduced by T3 (Liu et al. 2000; Machuca et al. 1995; Manchado et al. 2009) although, this modulation appears to be tissue-, subtype- and sex-specific. In fish, tissue-specific changes in *thr* expression after exogenous administration of T3 or antithyroid drugs has been previously described for a number of species including fathead minnow (Lema et al. 2009) and striped parrotfish (*Scarus iseri*) (Johnson and Lema 2011). However, those studies have focused on tissues such as liver, brain and gonads and up to our knowledge, no other study has assessed changes in *thr* expression in immune organs. Here, treatments affected the expression of the THR subtype in a tissue-specific manner. Liver and whole immune organs (spleen and head kidney) presented little or no response. More specifically, treatments had no effect on whole head kidney *thrs* expression. In liver, only after 7 days *thra* was slightly downregulated by PTU while *thrb* was upregulated by T3. The relatively low responsiveness of hepatic *thrs* after thyroidal manipulation with T3 or the antithyroidal drug methimazole (MMI) has been previously reported in striped parrotfish (Johnson and Lema 2011). In that study, exposure to T3 for 3 days provoked a sex-specific 2-fold upregulation of *thrs* in males while females exhibiting no effect. In our study, exposure to T3 for 7 days provoked an upregulation in both splenic *thra* and *thrb* while PTU had no effect. These results are difficult to contextualize as no other study has assessed changes in *thr* expression in piscine spleen, and although further studies should characterize the physiological significance, it may suggest a role for T3. For instance, in murine models, it has been shown that T3 acting through the *thra* is necessary for implementing erythropoiesis in the neonatal spleen (Angelin-Duclos et al. 2005).

On the contrary to whole organs, isolated immune cells from the head kidney and blood exhibited strong changes in *thrs*. In this regard, two points shall be highlighted. First, *thr* expression in isolated leukocytes responded much faster to the treatments (day 7) than those in the liver (day 15). Although it is too early to speculate about the functional implications, this may also suggest a particular role of thyroid signaling in trout leukocytes. In second place, the remarkable differences between whole immune organ and isolated cells emphasize the need of using pure isolates of the target cell population.

3.3. Does TH status affect the dynamics of leukocyte populations? Effects of T3/PTU on transcript levels of leukocyte markers in isolated immune cells.

A final question in this study was whether changes in thyroid status affect leukocyte composition. In mammals, it has been shown that different immune cell subpopulations present important differences in THR expression (Diehl et al. 2011)and this has consequences for thyroid-mediated alterations of immune cell differentiation and proliferation. For instance, THRA knockout mice (*Mus musculus*) exhibit impaired B-cell development (Arpin et al. 2000). In this final part of the work we tried to gain some insight on whether changes in thyroid status affect leukocyte composition. In order to do so, we measured several marker genes of macrophages, T lymphocytes (total, helper and suppressor) and B lymphocytes. The effects were tissue- and time-dependent, making very difficult establishing correlations or drafting solid conclusions. The complex relationships between thyroid hormones and immune cells have been previously reported, with studies showing contradictory results regarding the effect of hypo and hyperthyroidism on immunity (Rev. in De Vito et al. 2011). Specifically, previous studies in rats have shown that effects of hyper and hypothyroidism over white cells are tissue and organ dependent and opposite effects have been described even in the same study. For instance, PTU was found to differently alter helper (cd4+)/suppressor (cd8α+) T cells ratio, with increasing proportions in the spleen but decreasing indexes in the blood (Pacini et al. 1983). In another study, cd8α+ were found to decrease in hypothyroidism and increase in hyperthyroidism conditions when measured in blood. However in the spleen, differences were not significant (Ohashi and Itoh 1994). In hypophysectomized trout, a decrease in the Ig-producing leukocytes, suggesting an inactivation of B-cell differentiation, was observed in the head kidney but not in blood (Yada and Azuma 2002). Generally, it seems that in mammals the maintenance of the ratios of lymphocyte subpopulations is strongly modulated by T3 levels (Hodkinson et al. 2009). Similarly, in rainbow trout we have observed altered ratios of the marker genes after manipulation of TH status, which could be taken as a direct thyroid hormone effect on immune cell dynamics. However, the fact that T3 and PTU tended to induce similar effects could be indicative of indirect, not THR-mediated effects.

Although with this study we have demonstrated the presence of THRs in immune organs and cells of rainbow trout suggesting that thyroid signaling occurs, future work should address if, and under what specific conditions, immunomodulatory effects of the thyroid system are relevant with regard to the environmental exposure to thyroid-disrupting compounds.

V.CONCLUSIONS

CONCLUSIONS

- When performing environmental monitoring, only an appropriate combination of biological and chemical techniques allows the detection of the presence of trace levels of contaminants in a theoretically non affected waterbody.

- The approach followed in this work, based on the combination of several techniques, evidenced the ubiquitous presence of low concentrations of contaminants in areas with very little anthropogenic pressure, suggesting the widespread occurrence of pollution.

- The active biomonitoring (ABM) approach is a very simple and useful tool to demonstrate exposure to pollutants at low concentrations and recovery of animals.

- Farmed fish, which are maintained under known and constant environmental conditions, can be used as sentinels for detecting the presence of trace levels of contaminants and could serve as early-warning systems.

- Rainbow trout is a very useful species in environmental monitoring. Despite of not being an autochtonous species in Spain, its sensitivity to pollution together with the existence of a great body of literature with reference values, makes of it a very valuable organism to monitor pollution.

- Commercial fish feeds commonly applied in aquaculture are a potential source of EDCs. Further studies should address the effects of long-term feeding.

- The HER-LUC cell line, stably transfected with the seabass estrogen receptor, proved to be a good tool to assess estrogenicity in complex environmental samples.

- Although until now, much more attention has been paid to ER-mediated endocrine disruption, our results suggest that THR-mediated endocrine disruption also raises concern. Efforts should be done to elucidate the underlying mechanisms as well as the potential deleterious effects onto wildlife.

- The presence of THRs in immune organs and cells of rainbow trout suggests that thyroid signaling plays a role in the regulation of the immune activity in this piscine species.

- The greater expression of THRA in the immune-related organs of rainbow trout compared to e.g. liver, pinpoints to the notion that this receptor subtype is the main mediator of the effects of THs on rainbow trout immune cell function.

CONCLUSIONES

- Al llevar a cabo tareas de monitorización ambiental en cuerpos aucáticos teóricamente pristinossólo una combinación apropiada combinación de técnicas biológicas y químicas permite la detección de niveles traza de contaminantes.

- La aproximación aquí seguida, basada en la combinación de distintas técnicas, evidenció la presencia ubicua de concentraciones traza de contaminantes en áreas con poca presión antropogénica, lo cual es indicativo de una existenciageneralizada de contaminación.

- La aproximación conocida como “Biomonitorización activa” es una herramienta simple y muy útil para demostrar la exposición a contaminantes en bajas concentraciones así como para la recuperación de los animales.

- Los peces cultivados, que están mantenidos en condiciones ambientales conocidas y constantes, pueden ser usados como centinelas para detectar la presencia de niveles traza de contaminantes y por tanto sirven como sistemas de alerta temprana.

- La trucha arcoiris es una especie muy útil en monitorización ambiental. A pesar de no ser una especie autóctona de España, su sensibilidad a la contaminación, junto con la existencia de una gran cantidad de literatura con valores de referencia, la convierte en un organismo muy valioso en la monitorización ambiental.

- Los piensos comerciales utilizados comúnmente en acuicultura son una fuente potencial de disruptores endocrinos (DEs). En el futuro, nuevos estudios deberían abordar el efecto de su consumo a largo plazo.

- La línea celular HER-LUC, transfectada de manera estable con el receptor de estrógenos de lubina, ha demostrado ser una buena herramienta para valorar estrogenicidad en muestras ambientales complejas.

- Aunque hasta ahora la mayoría de estudios se han centrado en estudiar la disrupción endocrina mediada por el receptor de estrógenos, nuestros resultados sugieren que la disrupción endocrina mediada por el receptor de hormonas tiroideas (THR) también tiene una trascendencia importante. Se deben realizar esfuerzos para elucidar los mecanismos celulares subyacentes a este tipo de alteración endocrina así como sus efectos potencialmente perjudiciales en poblaciones naturales.

A.Q-G.

- La presencia de THRs en los órganos y células inmunes de la trucha arcoíris sugiere que la señalización tiroidea juega un papel en la regulación de la actividad inmune en esta especie.

- La mayor expresión del THRA en los órganos y células inmunes de la trucha arcoiris en comparación con otros tejidos (hígado), apunta a la idea de que este subtipo de receptor es el principal mediador de los efectos de las hormonas tiroideas sobre la función inmune de esta especie.

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APPENDIX

OTHER PEER-REVIEWED RESEARCH ARTICLES.

- Valdehita, A., Quesada-García, A., Delgado, M.M., Martín, J.V., García-González, M.C., Fernández-Cruz, M.L., Navas, J.M. 2014. In vitro assessment of thyroidal and estrogenic activities in poultry and broiler manure. *Science of the Total Environment* 472: 630-41.

- Connolly, M., Fernández- Cruz, M.L., Quesada-García, A., Alté, L., Segner H., Navas, J.M. Comparative cytotoxicity study of silver nanoparticles (AgNPs) in a variety of rainbow trout cell lines (RTL-W1, RTH-149, RTG-2) and primary hepatocytes. *Accepted for publication in the International Journal of Environmental Research and Public Health.*

- Quesada-García, A., Encinas, P., Valdehita, A., Baumann, L., Segner, H., Coll, J.M., Navas, J.M. T3 and PTU differentially affects transcript profile immune responses in rainbow trout head kidney. *In preparation.*